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Application of: Charlotte Kensil

Confirmation No.: 2171

Application No.: 09/760,506

Art Unit: 1636

Filed: January 12, 2001

Examiner: Qian, Celine X.

For: INNATE IMMUNITY-
STIMULATING COMPOSITIONS
OF CPG AND SAPONIN AND
METHODS THEREOF

Attorney Docket No.: 8449-153

SECOND DECLARATION OF DR. CHARLOTTE KENSIL UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Charlotte Kensil, Ph.D., do declare and state that:

1. I presently hold the position of consultant at Antigenics Inc. Antigenics Inc. is the owner of the entire right, title and interest in, to and under the invention described and claimed in the above-identified patent application.

2. I received a Ph.D. from the University of California, San Diego in 1981. My academic and technical experience and honors, and a list of my publications, are set forth in my *curriculum vitae*, attached hereto as Appendix 1.

3. I am the sole inventor of the invention described and claimed in the above-identified U.S. Application No. 09/760,506 ("the '506 application"). I have read and understand the '506 application. I have also read the Office Action dated April 20, 2004, including the references cited by the Examiner. I understand that an issue relevant to the rejection under 35 U.S.C. § 112, first paragraph for lack of enablement is whether any type of *Quillaja saponaria*

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saponin, whether in modified or unmodified form, can be used to treat cancer. I present this declaration to address this issue.

4. I will discuss why *Quillaja saponaria* saponins generally, *i.e.*, not just QS-21, are expected to be useful to treat any type of cancer. In particular, *Quillaja saponaria* saponins share a common structure that gives rise to the common innate immunity function, and thus would be expected to function in the same manner with respect to increasing innate immunity and treating cancer. For example, QS-7, QS-17, QS-18, and QS-21 are saponins that are derived from *Quillaja saponaria*. All are structurally very similar. Kensil *et al.* (1993, "Novel Adjuvants from *Quillaja saponaria* Molina" in AIDS Research Reviews Volume 3 edited by Koffet *et al.* New York; attached hereto as Exhibit 1, "Kensil I") shows the structures of QS-17, QS-18, and QS-21 in Figure 2, derived from comparison of monosaccharide composition and molecular weight. Kensil *et al.* (1998, *Dev Biol Stand* 92: 41-47, attached hereto as Exhibit 2, "Kensil II") shows the structures of QS-7 and QS-21 in Figure 1. Compared to the large portion of the structure that is identical, any differences between QS-7, QS-17, QS-18, and QS-21 are minor.

5. All *Quillaja saponaria* saponins whose structure is known to me share two structural features - a triterpene backbone and a 2,3, glucuronic acid carboxyl group (*see* the paragraph spanning pages 1403 and 1404 of Soltysik *et al.*, 1995, *Vaccine* 13:1404-10, attached hereto as Exhibit 3, "Soltysik"). Modification of the triterpene aldehyde of the backbone inactivated the ability of QS-21 to stimulate immune response as manifested by antibody production and cytotoxic T lymphocyte activity (page 1408, second column, first full paragraph of Soltysik (Exhibit 3)).

6. Furthermore, evidence demonstrates that all *Quillaja saponaria* saponins (even those additional to QS -17, -18, and -21) have very similar structures. Mild alkaline hydrolysis of a crude extract of *quillaja* bark containing *Quillaja saponaria* saponins generates only two major structures: desacysaponin -1 and -2 (DS-1 and DS-2) (*see* Higuchi *et al.*, 1987, *Phytochemistry* 26:229-235, attached hereto as Exhibit 4, "Higuchi"). DS-1 was shown to contain glucuronic acid, galactose, xylose, fucose, rhamnose, apiose, and quillaic acid, whereas

DS-2 contained these same components plus glucose (see Higuchi). Thus, DS-1 and DS-2 have very similar structures, differing only in whether or not glucose is present.

7. Unlike the majority of saponins from species other than *Quillaja saponaria*, all *Quillaja saponaria* saponins are acylated. QS-17, QS-18 and QS-21 are acylated at identical positions and removal of this acyl group decreases immune adjuvant activity as reflected by total IgG response as compared to acylated counterparts (see page 2808, paragraph spanning the first and second columns of Liu *et al.*, 2002, *Vaccine* 20:2808-15, attached hereto as Exhibit 5, "Liu" (Liu shows that deacylated QS-21 was inactive in inducing IgG2a and cytotoxic T lymphocyte (CTL) responses); and Kensil *et al.*, *Vaccines* 92, Cold Spring Harbor Laboratory Press pp. 35-40 (1992), attached hereto as Exhibit 6, "Kensil III"). Thus, there are specific structural components (of QS saponins) that influence immune adjuvant activity.

8. With respect to common function, a characteristic shared in common by saponins QS-7, QS-17, QS-18, and QS-21 in regard to enhancement of adaptive immunity is their ability to stimulate IgG2a responses in mice (see Kensil *et al.*, 1991, *J Immunol* 146: 431-437, attached hereto as Exhibit 7, "Kensil IV"). Although more similar structurally than different, QS-7 and QS-21 represent two extremes within the class of *Quillaja saponaria* saponins, e.g., QS-7 has a short acyl chain whereas QS-21 has a long acyl chain; QS-7 is more heavily glycosylated than QS-21. Despite these slight structural differences, QS-7 and QS-21 both stimulate innate immune responses, for example, by enhancing natural killer cell lytic activity (see the specification of the above-identified patent application at page 7 and Figure 3) although to different levels dependent upon dose. Activated natural killer cells also produce the cytokine interferon gamma (see Abbas *et al.*, in *Cellular and Molecular Immunology*, WB Saunders Company, Philadelphia, PA, 1997, p. 269, first full paragraph, attached hereto as Exhibit 8). Snapper and Paul show that interferon gamma stimulates the expression of IgG2a and inhibits the production of isotypes such as IgG1 in mice (see Snapper *et al.*, 1987, *Science* 236: 944-947, abstract, attached hereto as Exhibit 9). Hence, a characteristic effect on adaptive immunity (enhanced IgG2a response) seen within the class of saponins encompassed by QS-7, QS-17, QS-18, and QS-21 was also seen for QS-7 and QS-21 for the activation of a cell type (natural killer cells) that produce the cytokine (interferon gamma) that mediates that characteristic adaptive immunity effect. Hence, the ability of QS-17 and QS-18 to induce an IgG2a response also

indicates that these saponins influence the activation of natural killer cells resulting in induction of interferon gamma by the innate immune system.

9. Because of the structural similarity of the *Quillaja saponaria* saponins and the correlation of structure with function, I conclude that usefulness in increasing innate immunity and treating cancer is reasonably expected to be a general property of the genus of *Quillaja saponaria* saponins.

10. As support for the Examiner's contention that it is unpredictable whether any *Quillaja saponaria* saponin, modified or unmodified, can treat cancer, the Examiner cites Rao and Sung, 1995, *J. Nutr.* 125:717S-724S ("Rao") as teaching that the different structures of saponins affect their biological activity.

11. While Rao does indicate that the structure of saponin can affect its biological activity, the Examiner's statement disregards the teachings of Kensil IV. Firstly, Figure 3 of Kensil IV showed that all four *Quillaja saponaria* saponins, i.e., QS-7, -17, -18, and -21, induced similar antibody (IgG) titers (with overlapping error bars) in mice. Secondly, Table 1 of Kensil III demonstrated that all four *Quillaja saponaria* saponins, i.e., QS-7, -17, -18, and -21, induced IgG2a titers, at levels higher than other adjuvants used. It is not common for adjuvants to induce IgG2a responses. Therefore, the ability of four structurally related *Quillaja saponaria* saponins to induce an IgG2a response is further proof of the similarities in their immune adjuvant activity. Moreover, as discussed above, the ability of four structurally related *Quillaja saponaria* saponins to induce an IgG2a response is also indicative of their ability to increase the innate immune response. The fact that QS-7, -17, -18, and -21 have different structures and may induce antibody responses with small quantitative differences is irrelevant to the issue at hand. Firstly, the Examiner's statement ignores the fact that those *Quillaja saponaria* saponins have a common structure that gives rise to their common innate immunity activity (see ¶¶ 4-9, hereinabove). Secondly, while small quantitative differences in their immunologic activities may exist, all *Quillaja saponaria* saponins are expected to have qualitatively similar immunostimulatory activity, and thus are reasonably predicted to be effective in treating cancer. The point is that there are specific structural components, such as a triterpene backbone, a 2,3, glucuronic acid carboxyl group, and an acyl group at certain positions, shared by the *Quillaja saponaria*

saponins, that are required for or influence their immunologic activity. The minor structural differences in the *Quillaja saponaria* saponins do not qualitatively affect the immunologic activity of these compounds.

12. Moreover, the teachings of the prior art provide guidance in terms of what kinds of modifications can be made to *Quillaja saponaria* saponins without adversely affecting their immunologic activity, including the ability to induce IgG2a. Soltysik teaches that derivatives of QS-21 containing a modification of a carboxyl group on glucuronic acid induced antibody titers at levels similar to QS-21. Moreover, IgG2a levels increased according to the same dose response curves as for total IgG. See Soltysik, abstract and page 1407 (Exhibit 3). Thus, the prior art teaches modified *Quillaja saponaria* saponins having similar immunologic activity to unmodified *Quillaja saponaria* saponins. These types of modified *Quillaja saponaria* saponins would be predicted to be effective in treating cancer.

13. I hereby declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: October 20, 2004

Charlotte Kensil
Charlotte Kensil, Ph.D.

CURRICULUM VITAE

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DATE AND PLACE OF BIRTH: May 15, 1954, Fairbury, Illinois

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EDUCATION:

Institution and Location	Degree Awarded	Year	Scientific Field
University of Illinois Champaign-Urbana, Illinois	B.Sc.	1976	Biochemistry
University of California San Diego, California	Ph.D	1981	Chemistry

PROFESSIONAL EXPERIENCE:

<u>Date</u>	<u>Position</u>
6/81-12/85	Postdoctoral fellow. Biochemistry Department, University of Connecticut Health Center, Farmington, CT.
1/86-1/88	Staff Scientist, Protein Chemistry Department, Cambridge BioScience Corporation, 365 Plantation St., Worcester 01605.
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11/00-3/03	Vice-president of Research, Antigenics Inc, 175 Crossing Boulevard, Framingham, MA 01702

RESEARCH PUBLICATIONS:

1. Rafi-Janajreh, A., Tongren, J.E., **Kensil, C.R.**, Hackett, C., Candal, F., Lal, A., Udhayakumar, V. 2002. Influence of adjuvants in inducing immune responses to different epitopes included in a multiepitope, multivalent, multistage *Plasmodium falciparum* candidate vaccine (FALVAC-1) in outbred mice. *Exp. Parasitology*. 101: 3-12.
2. Liu, G., Anderson, C., Scaltreto, H., Barbon, J., and **Kensil, C.R.** 2002. QS-21 structure/function studies: Effect of acylation on adjuvant activity. *Vaccine*. 20: 2808-2815.
3. Waite, D.C., Jacobson, E.W., Ennis, F.A., Edelman, R., White, B., Kammer, R., Anderson, C., and **Kensil, C.R.** 2001. Three double-blind randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21. *Vaccine*. 19 (28-29): 3957-3967.
4. Gozar, M.M., Muratova, O., Keister, D.B., **Kensil, C.R.**, Price, V.L., and Kaslow, D. 2001. *Plasmodium falciparum*: Immunogenicity of Alum-adsorbed clinical-grade TBV25-28, a yeast-secreted malaria transmission-blocking vaccine candidate. *Exp. Parasitology* 97(2): 61-69.
5. Boyaka, P.N., Marinaro, M., Jackson, R.J., van Ginkel, F.W., **Kensil, C.R.**, and McGhee, J.R. 2001. Oral QS-21 requires early IL-4 help for induction of mucosal and systemic immunity. *J. Immunology*, 166: 2283-2290.
6. **Kensil, C.R.** 2000. QS-21 Adjuvant. In: Methods in Molecular Medicine, Vol 42: Vaccine Adjuvants: Preparation Methods and Research Protocols (O'Hagan, D.T. ed.), Humana Press, Inc., Totowa, NJ, pp 259-271.
7. **Kensil, C.R.** and Kammer, R. 1998. QS-21: a water-soluble triterpene glycoside adjuvant. *Exp. Opin. Invest. Drugs* 7(9): 1475-1482.
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U.S. PATENTS:

1. Saponin Adjuvant.
Inventors: **Charlotte A. Kensil** and Dante J. Marciani
Assignee: Antigenics Inc., Framingham, MA
Patent Number: 5,057,540
Issued: Oct. 15, 1991
2. Methods for Enhancing Drug Delivery with Modified Saponins
Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
Assignee: Antigenics Inc., Framingham, MA
Patent Number: 5,273,965
Issued: Dec. 28, 1993
3. Vaccine Comprising Recombinant Feline Leukemia Antigen and Saponin Adjuvant
Inventors: Gerald Beltz, Dante J. Marciani, C.-H. Hung, and **Charlotte A. Kensil**
Assignee: Antigenics Inc., Framingham, MA
Patent Number: 5,352,449
Issued: Oct. 4, 1994
4. Modified Saponins for Enhancing Drug Delivery
Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
Assignee: Antigenics Inc., Framingham, MA
Patent Number: 5,443,829
Issued: Aug. 22, 1995
5. Saponin-Antigen Conjugates and the Use There of
Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
Assignee: Antigenics Inc., Framingham, MA
Patent Number 5,583,112
Issued: Dec. 10, 1996
6. Drug Delivery Enhancement via Modified Saponin
Inventors: **Charlotte A. Kensil**, Sean Soltysik, Dante J. Marciani, and Joanne Recchia
Assignee: Antigenics Inc., Framingham, MA
Patent Number 5,650,398
Issued: July 22, 1997
7. Saponin Adjuvant Composition
Inventor: **Charlotte A. Kensil**
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Novel Adjuvants from *Quillaja saponaria* Molina

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INTRODUCTION

Modulation of immune responses to HIV-1 vaccines, particularly subunit vaccines, may require the development of new adjuvants. One such adjuvant can be found in a unique group of compounds, the plant saponins. In particular, certain saponins from *Quillaja saponaria* Molina are potent stimulators of immune responses. The biological activities of crude and partially purified extracts from *Quillaja saponaria* have been described in numerous published reports. Unfortunately, due to lack of purification, it was not clear which components of the extracts were responsible for these biological activities. We have purified the major adjuvant active components from *Q. saponaria*, allowing concurrent structure and function characterization. In this chapter, we review the immunological and structural characterization of this important new adjuvant class, with particular focus on QS-21, the best characterized of the *Q. saponaria* adjuvants.

Quillaja saponaria Molina is a species of tree indigenous to South America. The bark of this tree is rich in triterpene glycoside saponins, representing up to 10% of the weight of the bark. The bark extracts have numerous industrial uses for which surface active agents are needed. As such, the bark is an export product of several countries, such as Chile. The extracts of *Q. saponaria* have also been shown to contain components useful in vaccine applications.

Extracts of the bark of *Quillaja saponaria* were first shown to enhance the protective effect of a foot-and-mouth-disease vaccine in cattle (1). However, these crude saponin extracts consisted predominantly of tannins in addition to the saponin fraction. In 1974, Dalsgaard (2) isolated the saponin fraction away from the contaminating tannins and showed that all of the adjuvant activity was contained in the saponin fraction. He further purified this fraction by a combination of gel filtration and ion-exchange chromatography to produce a fraction that is now known as Quil A. Quil A has been utilized extensively as an immunological adjuvant for T-dependent antigens (2,3). It is also a critical component of immunestimulating complexes (ISCOMs) (4).

Quil A was shown to be a very heterogeneous mixture (5), consisting of at least 20 peaks when analyzed by reverse-phase high-performance liquid chromatography (HPLC). Attempts to purify these components to homogeneity in aqueous systems were ineffective, although partial resolution of adjuvant activity away from irritating substances was achieved by gel-filtration chromatography (6). The difficulty in purification was due to the detergent nature of the saponin fraction; mixed micelle formation between the different saponins and other lipophilic contaminants prevented effective separation in aqueous solution.

We have recently identified the adjuvant active components from *Q. saponaria* (7). In our study, the total saponin fraction from *Q. saponaria* was resolved into individual, distinct saponins by disruption of the micellar interactions in organic solvent and subsequent purification by HPLC. These purified components were evaluated for adjuvant activity in mice using bovine serum albumin (BSA) as the immunogen and by measuring increases in antibody titers. The four predominant peaks in the saponin fraction were identified as adjuvant active components; these peaks were designated QS-7, QS-17, QS-18, and QS-21. The purification of these distinct saponins has allowed us to initiate studies to characterize the purified saponins both immunologically and chemically.

STIMULATION OF ANTIBODY RESPONSES

Increases in antigen-specific IgG titers in mice in response to formulations containing QS-21 as adjuvant have been observed with the antigens BSA (7), cytochrome b₅ (7), and ovalbumin (OVA) (8). The antibody titers induced in mice by two intradermal immunizations with 20 µg of purified saponin with either the antigen cytochrome b₅ (7) or OVA (data not shown) were found to be comparable to the titers

induced by Freund's complete adjuvant and were higher than the titers induced by aluminum hydroxide.

The dose effect of QS-7, QS-17, QS-18, and QS-21 on the stimulation of antigen-specific IgG was assessed in both CD-1 and C57BL/6 mice. Two immunizations of formulations containing BSA or OVA and varying doses of these purified saponins (over a dose range up to 40 μ g with QS-18 and up to 80 μ g with QS-7, 17, and 21) were given to group of five mice (7-9). The minimum optimal dose was determined to be approximately 5 μ g each of these saponins; the antibody titers reached a maximum with purified saponin doses of 5 μ g and higher.

IgG SUBCLASS SWITCHING

Adjuvants are known to regulate IgG subclasses (10), a factor that is an important component of an adjuvant's potential protective role against viral and bacterial infections. Mouse IgG₂ subclasses fix complement at a higher level than IgG₁ (11). Additionally, IgG_{2a} and IgG_{2b} bind strongly to Fc receptor, a response critical to antibody-dependent cellular cytotoxicity (12). Hence, the ability of an adjuvant to change an antibody response from predominantly an IgG₁ response to a broadened response containing IgG₁, IgG_{2b}, and IgG_{2a} may be an essential component of protective responses to pathogens.

The purified saponins clearly induce subclass switching. Immunization of CD-1 mice with the antigen cytochrome b₅ and QS-7, QS-17, QS-18, or QS-21 broadens the antibody response to include high levels of IgG_{2a} and IgG_{2b} isotypes compared to immunization with antigen alone (7). An example of the isotype switching with another T-dependent antigen, OVA, is shown in Table 1. OVA-specific IgG₁, IgG_{2b}, and IgG_{2a} are significantly increased after three intradermal immunizations of C57BL/6 mice with OVA and QS-21 compared to immunization with OVA alone. The IgG subclass switching has also been demonstrated with BSA/QS-21 and with recombinant FeLV gp70/QS-21 and HIV-1 gp160/QS-21 vaccines (data not shown). The induction of IgG_{2a} suggests the possible activation of Th₁ cells and subsequent

Table 1 Influence of QS-21 on Antibody Titers to T-Dependent Antigen

Formulation*	IgG ₁	IgG _{2b}	IgG _{2a}
OVA (25 μ g)	140	<10	<10
OVA (25 μ g)	9100	2000	2400
QS-21 (10 μ g)			

*C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10, and 12 weeks of age with the indicated formulations. Sera were collected 2 weeks after the last immunization and were analyzed for antibody to ovalbumin by EIA.

production of the cytokine interferon- γ as part of the cascade induced by the saponin adjuvants; this cascade has been proposed by Karagouni et al. (10), who also noted a stimulation of IgG_{2a} production in response to immunization with crude saponin.

LACK OF INDUCTION OF IgE

Some crude saponin preparations have induced allergic responses mediated by the production of IgE (13). However, the purified saponins QS-7, -17, -18, and -21 did not induce IgE with the antigen cytochrome b₅ (7), suggesting that a nonsaponin component(s) of the crude preparations was responsible for stimulation of IgE titers. The low levels of IgE measured experimentally are consistent with the high levels of IgG_{2a} elicited by these purified saponin adjuvants. Typically, induction of IL-4 [shown to be correlated with production of IgE antibody in mice (14)] is inversely correlated with induction of interferon- γ , which is associated with the production of IgG_{2a} (15).

INDUCTION OF IMMUNOLOGICAL MEMORY

Also critical to protective immune response is the induction of immunological memory. Not all adjuvants invoke memory responses. Studies with recombinant HIV-1 glycoproteins in BALB/c mice indicate that the saponin QS-21 is effective in inducing anamnestic responses. BALB/c mice immunized twice with 10- μ g doses of recombinant HIV-1 gp160/aluminum hydroxide/10 μ g of QS-21 responded with significant increases in antibody titers 1 week after challenge with inactivated HIV-1 IIIB, whereas mice immunized with the same formulation without QS-21 did not respond (16). The kinetics of the rapid titer increase are consistent with a memory response. Memory responses were also observed to an FeLV vaccine containing recombinant FeLV gp70, aluminum hydroxide, and QS-21. Specific pathogen-free cats immunized twice with this vaccine developed a rapid neutralizing response in response to intraperitoneal challenge with infectious FeLV (17).

QS-21 AS ADJUVANT FOR T-INDEPENDENT ANTIGENS

Flebbe and Braley-Mullen (3) demonstrated that Quil-A is an effective adjuvant for a hapten coupled to the T-independent antigens Ficoll, lipopolysaccharide (LPS), and *Brucella abortus*. Because of the heterogeneity of Quil A, it was not clear which component(s) was responsible for this activity. At least one purified saponin, QS-21, has been shown to significantly augment antibody response to the T-independent antigen *E. coli* 055:B5 polysaccharide (O-PS) (18), prepared by acid hydrolysis of phenol-extracted LPS. This response was not due to residual native LPS activity, because similar results were observed in the LPS nonresponder mouse strain C3H/HeJ. The antibody responses raised to native LPS after two intradermal immunizations with

Table 2 Influence of QS-21 on Antibody Titers to T-Independent Antigen

Formulation*	IgG ₁	IgG _{2b}	IgG _{2a}
O-PS (100 µg)	15,000	3900	9800
O-PS (100 µg)	82,000	99,000	115,000
QS-21 (15 µg)			

*C3H/HeJ mice (19 per group) were immunized intradermally at 8 and 10 weeks of age with the indicated formulations. Sera were collected 1 week after the last immunization and were analyzed for antibody to native *E. coli* lipopolysaccharide by EIA.

O-PS and 15 µg of QS-21 are described in Table 2. Significant increases in LPS-specific IgG₁, IgG_{2a}, and IgG_{2b} were observed when compared to O-PS alone.

EFFECT ON CELL-MEDIATED IMMUNE RESPONSES

One unique and important immune modulation property of the QS-21 adjuvant is its ability to induce antigen-specific, class I major histocompatibility complex (MHC) antigen-restricted, CD8+ cytotoxic T lymphocytes (CTLs) when used in viral subunit vaccine formulations. This property was investigated using soluble OVA or OVA adsorbed onto aluminum hydroxide as immunogens (8). C57BL/6 mice that were immunized with soluble native or denatured OVA in formulations that contained increasing quantities of QS-21 raised CTL responses that were specific for the immunodominant epitope of OVA (OVA₂₅₈₋₂₇₆). Similar responses were induced using aluminum hydroxide-adsorbed OVA when mixed with the QS-21 adjuvant, but not when the aluminum hydroxide-adsorbed OVA was used alone (Figure 1). The CTL activity was totally destroyed by treatment in vitro with monoclonal antibody specific to the CD8 antigen plus complement.

Comparison testing of experimental HIV-1 vaccines containing aluminum hydroxide-adsorbed recombinant HIV-1 gp160 protein and the QS-21 adjuvant to formulations containing only the aluminum hydroxide-adsorbed gp160 has been conducted using BALB/c mice and rhesus macaques. Cell-mediated immune responses were increased similarly, and this included the recognition of additional epitopes. Class I MHC antigen-restricted CTLs that were specific for the V3 loop were also raised but, again, only if the QS-21 adjuvant was part of the formulation. Testing using rhesus macaques has demonstrated that the QS-21 is functionally active and safe for use in primates (20).

STRUCTURE OF QUILLAJA SAPONINS

A full understanding of the mechanism of action of these purified saponins requires an understanding of their structures. Studies on alkaline hydrolysis products isolated from

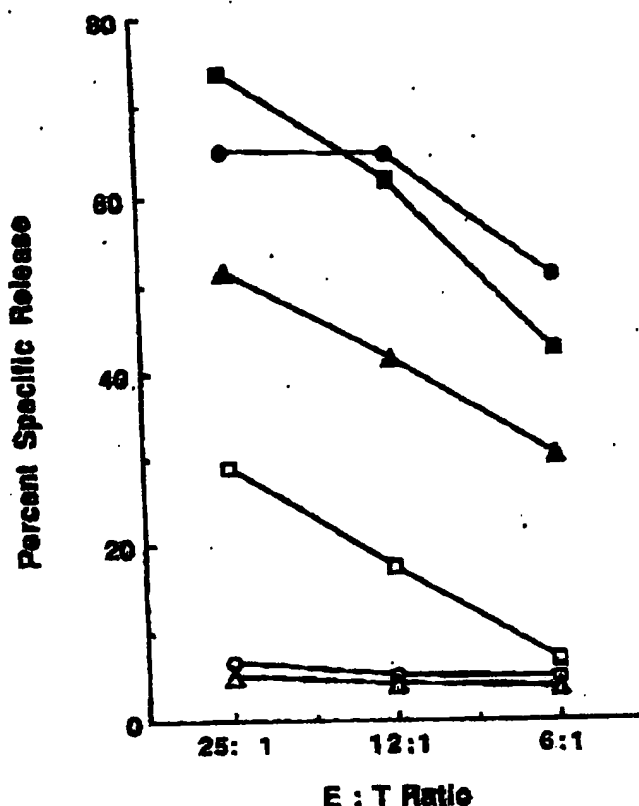


Figure 1 CTL activity measured using EL4 cells expressing OVA as target cells [E.G7-OVA cells (19)] and splenic mononuclear cells obtained from C57BL/6 mice following three immunizations with native soluble OVA (○), denatured soluble OVA (△), or OVA adsorbed to alum (□) and the same formulations mixed with 20 µg/dose of QS-21 (shown as solid symbols). Maturation of precursor CTL to functional effector cells *in vitro* was effected using irradiated E.G7-OVA cells (8). Responses are shown as "percent specific lysis" using a titration of effector to targets (E:T ratio).

crude extracts of *Q. saponaria* showed that the predominant components were quillaic acid 3,28-O-bisglycosides (21,22). An intact saponin, designated QS-III, was isolated and determined to be a quillaic acid 3,28-O-bisglycoside with a fatty acid in ester linkage to the 3 position of fucose [linked to C₂₈ of the triterpene (23)]. Determination of the monosaccharide compositions of QS-7, -17, -18, and -21 (7,9) indicated that these purified saponin adjuvants were closely structurally related to each other and to QS-III. Fast atom bombardment-mass spectroscopy indicated that the molecular weights of QS-17, QS-18, and QS-21 were 2321, 2174, and 2012, respectively, with the differences in molecular weight corresponding to an additional glucose in QS-18 compared to QS-21 and an additional rhamnose in QS-17 compared to QS-18. Figure 2 shows the proposed structures of QS-17, QS-18, and QS-21, derived from comparison of our monosaccharide composition and molecular weight

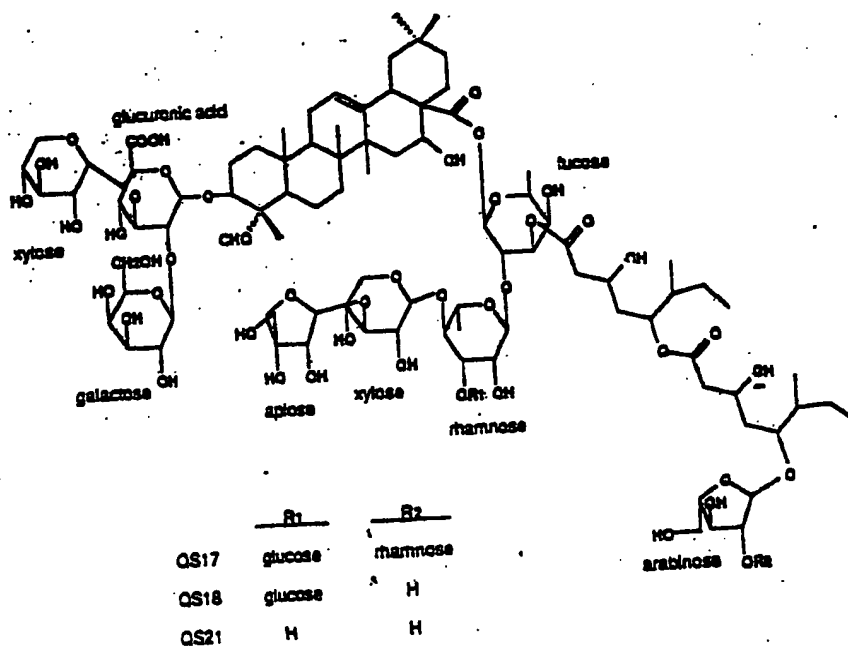


Figure 2 Proposed structure and relationship of QS-17, QS-18, and QS-21.

data for these purified saponins with those of the closely related saponin QS-III that was isolated and characterized by Higuchi. The structure of QS-7 is still unknown, although monosaccharide analysis indicates that its glycoside composition is related to that of the other major saponin adjuvants.

Several functional groups on the saponins have been shown to be important for immune response. Deletion of the fatty acid from either QS-18 or QS-21 substantially reduced the stimulation of antibody titer by the resulting truncated triterpene glycoside compared to the intact saponins [evaluated in CD-1 mice with the antigen BSA (9)]. Periodate oxidation of QS-18 (containing two periodate-sensitive monosaccharide residues, galactose and apiose) destroyed the capacity of this saponin to stimulate antibody titers to BSA, indicating that one or both of these residues are critical to the adjuvant activity of this saponin (9). Studies are under way to identify other critical sites on the saponin adjuvants. It is possible that separate sites on these molecules are responsible for the antibody stimulation and the CTL-stimulation activities.

FORMULATIONS

The vaccine formulations of QS-21 and other purified saponins from *Q. saponaria* described in this review were soluble aqueous preparations prepared by simple admixture of saline solutions of purified saponin and antigen and were not prepared as

emulsions or ISCOMs. The saponins were shown to be effective as adjuvants at concentrations that are below the estimated critical micellar concentration (approximately 100 $\mu\text{g/ml}$ for QS-21 in phosphate-buffered saline pH 7.2), indicating that the micellar structure of the saponins is not critical to the adjuvant response.

We have also demonstrated that QS-21 can be successfully mixed into vaccine formulations that are already adjuvanted with aluminum hydroxide. QS-21 (at a dose of 10 μg) boosted total IgG titers by a factor of 10-fold when added to recombinant FeLV gp70 antigen absorbed with aluminum hydroxide (24) after a single intradermal immunization. Isotype switching from predominantly IgG₁ with aluminum hydroxide alone to IgG₁, IgG_{2b}, and IgG_{2a} in the QS21/aluminum hydroxide formulation was also observed with this antigen (25). This effect has also been observed with aluminum hydroxide-absorbed recombinant HIV envelope antigen (16), with total antigen-specific IgG titer increases of 25–125-fold in BALB/c mice after two immunizations with recombinant HIV-1 gp160 absorbed to aluminum hydroxide and 10 μg QS-21 compared to immunization with the aluminum hydroxide-absorbed protein only. Hence, the saponins may be utilized successfully in fully soluble vaccine formulations as well as in formulations adjuvanted with a solid-matrix adjuvant such as aluminum hydroxide.

We have previously suggested that a close association between the saponin adjuvant and the antigen is important for optimal immune response (7). Such association may occur through hydrophobic interaction between the amphipathic adjuvant and hydrophobic crevices on the protein antigen. Although we have seen clear adjuvant effects with antigens that do not bind QS-21, the adjuvant effect is higher when the antigen is denatured, allowing an increased binding of QS-21 (data not shown). Hence, we expect that a forced association of antigen and adjuvant through covalent linkage may improve immune response to small, hydrophilic proteins or peptides that ordinarily do not bind QS-21.

Similar approaches to vaccine formulation have been utilized successfully with the adjuvant muramyl dipeptide (MDP) (26,27). To investigate this type of formulation with the purified saponin adjuvants, we covalently coupled QS-21 to hen-egg lysozyme (9). This conjugation was carried out using carbodiimide chemistry to directly couple the QS-21 glucuronic acid to protein amino groups. A 1:1 molar conjugate was tested in C57BL/6 mice and compared to the same ratio of unconjugated lysozyme and QS-21. No antibody response was observed in these mice with the unconjugated lysozyme, consistent with this being a nonresponder strain to lysozyme. However, an antibody response was induced by the 1:1 molar conjugate. Still higher responses were induced when additional free QS-21 was added to the conjugate, suggesting that the conjugated QS-21 provided a binding site for additional QS-21 to bind to lysozyme, further enhancing the antibody stimulation. Hence, although with most antigens the purified saponin adjuvants will boost immune responses in unconjugated forms, vaccine formulations containing conjugated antigen and adjuvant show promise for boosting response to antigens that are otherwise not well-adjuvanted by the

saponins. We are currently testing immune responses to conjugates prepared from peptides and QS-21 to determine whether such formulations will be useful in vaccines against HIV-1.

TOXICITY OF DIFFERENT PURIFIED SAPONINS

One of the drawbacks of utilizing crude or partially purified saponins as an adjuvant is the toxicity associated with these preparations, which has been noted by several investigators (3,5). However, in a test of HPLC-purified saponins, it was found that the adjuvant active saponins covered a wide range of toxicity (assessed by lethality in mice); the saponin QS-18 was considerably more toxic than the original bark extract and the saponins QS-7 and QS-21 were considerably less toxic (7). No correlation was made between adjuvant activity and toxicity because both toxic and nontoxic saponins were adjuvant-active in a similar dose range in mice. In addition, no correlation was made between the hemolytic activity of the saponins and toxicity in mice (7).

It is important to note that the saponin QS-18, which is the most toxic component in mice, is the predominant saponin in all samples of *Q. saponaria* bark and commercial saponin samples that we have analyzed, suggesting that it may be responsible for much of the toxicity in mice that has been noted with these samples. Our approach to the use of saponins as adjuvants in vaccines is to use homogeneous saponin components with well-defined adjuvant potency and toxicity rather than partially purified extracts that may vary in proportions of toxic and adjuvant-active components. The ideal adjuvant candidate would be a saponin that is more potent as an adjuvant than the original extract but considerably less toxic. QS-21 shows considerable potential as such an adjuvant. Tests of QS-21 doses of 50 µg administered to rabbits by the intramuscular route showed that there was no observable effect on clinical hematology and serum chemistry data or on gross and microscopic pathology, after administration of four doses over a 2-week period (data not shown). Field tests of a commercial FeLV vaccine containing QS-21 as adjuvant showed that this vaccine was safe for use in cats (17); in addition, an experimental HIV-1 vaccine containing 50 µg QS-21 was used safely in primates (20).

SUMMARY

Individual saponins from *Quillaja saponaria* can be purified to homogeneity, allowing an immunological and structural characterization of the predominant adjuvant components from this species. One of these saponins, QS-21, has been tested extensively as an adjuvant in mice. It shows potent activity for stimulation of antigen-specific antibody titers to T-dependent antigens, including significant increases in IgG_{2a} and IgG_{2b}, as well as in IgG₁, augments class I MHC-restricted CTLs to subunit vaccines and, surprisingly, augments an increase in antibody titer to T-independent antigens.

The cellular mechanism by which QS21 evokes these responses is still unclear. Future studies will be directed to elucidation of this mechanism as well as of the sites on the QS-21 molecule required for the adjuvant responses.

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QS-21 and QS-7: Purified Saponin Adjuvants

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Key words: QS-21, QS-7, adjuvant, *Quillaja saponaria*, saponin, vaccine.

Abstract: QS-21 and QS-7 are two adjuvant-active saponins that can be obtained in high purity from *Quillaja saponaria* Molina extracts. QS-21 is a highly characterized compound and is known to be a potent adjuvant for antibody and CD8⁺ CTL response to subunit antigens. Less is known about the activity and structure of the hydrophilic saponin QS-7. Hence, we have carried out a detailed structural and immunological characterization. As with QS-21, QS-7 was shown to be a 3,28-O-bisglycoside quillaic acid, with some differences being a higher degree of glycosylation and a considerably shorter fatty acyl unit in QS-7. These differences were correlated to a lower lytic activity against sheep red blood cells. Different doses of QS-7 were evaluated for stimulation of immune response to the antigen ovalbumin, given three times by subcutaneous route to C57BL/6 mice. QS-7 doses of 40 µg or higher were shown to induce a strong CD8⁺ CTL response reproducibly against E.G7-OVA targets (similar to that induced by a 5-10 µg dose of QS-21). QS-7 (at doses above 5 µg) was also shown to stimulate CTL against peptide 18 of HIV-1₁₁₆ gp120 after three immunizations of Balb/c mice with recombinant gp120 and different doses of QS-7. These data suggest that a hydrophilic saponin with low lytic activity can stimulate MHC Class I CTL responses although a higher minimum dose may be required for some antigens.

INTRODUCTION

Aqueous extracts of the bark of the South American tree *Quillaja saponaria* Molina contain a potent immunological activity. These extracts are complex mixtures of tannins, polyphenols, and saponins. The adjuvant activity was shown to be associated with the saponin fraction [1]. This fraction consists of many diverse acylated bisdesmodic quillaic acid glycosides. Several predominant *Q. saponaria* saponins were HPLC-purified and identified as adjuvants [2]. One of these purified saponins, QS-21, has been characterized extensively as an adjuvant because of its potent adjuvant activity and low toxicity [3-5]. It is known to stimulate antibody and cytotoxic T lymphocyte responses to subunit vaccines in mice. QS-21 is currently under evaluation in clinical trials with various vaccine antigens [6, 7].

Other saponins from *Q. saponaria* are known to have adjuvant activity for the stimulation of antibody responses. A saponin known as QS-7 is of particular interest as a potential adjuvant. This saponin is more hydrophilic and less lytic to red

blood cells than the other major saponins. It was also shown to have low toxicity, with doses of 0.5 mg being tolerated well by mice. QS-7 was shown to be an adjuvant for antibody responses in mice to the antigens bovine serum albumin and beef liver cytochrome b₅ [2]. However, its potential as an adjuvant for cell mediated immune responses was not explored in previous studies. In this study, we have evaluated the adjuvant effect of QS-7 for induction of cytotoxic T lymphocytes to two antigens in mice, ovalbumin (OVA) and recombinant HIV-1 gp120.

MATERIALS AND METHODS

QS-7 and QS-21 were purified from an aqueous extract of *Q. saponaria* bark by reversed-phase HPLC. QS-21 and QS-7 were shown to be $\geq 98\%$ and $> 95\%$ pure, respectively, when analyzed by reversed phase HPLC analysis on Vydac C4 [5]. The deacylsaponins were prepared by alkaline hydrolysis of aqueous solutions of QS-7 or QS-21, followed by HPLC to isolate the deacylated saponin. Fast atom bombardment mass spectroscopy of purified saponins was carried out at MScan Corp., Westchester, PA. Carbohydrate analysis was carried out by Complex Carbohydrate Corp., Athens, GA.

Immunization was carried out with the antigen OVA in female C57BL/6 mice or with the antigen HIV-1_{IIIIB} gp120 in female Balb/c mice. OVA (grade VI) was obtained from Sigma (St. Louis, Missouri). Purified recombinant HIV-1_{IIIIB} gp120, expressed by a baculovirus vector in insect cells, was produced as described previously [8]. All mice were 8-10 weeks of age at the start of the first immunization. The antigen doses were 25 μ g. Adjuvant doses were varied between 0 to 80 μ g. All vaccines were administered by the subcutaneous route at days 0, 14, and 28 of the protocol. Immunological analysis was carried out on splenocytes obtained between days 42 and 56. Cytotoxic T lymphocyte responses were determined as described previously [3]. Haemolytic activity was determined by lysis of sheep red blood cells (SRBC, BioWhittaker, Inc., Walkersville, MD) as described previously [2].

RESULTS

Structural analysis

QS-7 was analysed by fast atom bombardment mass spectroscopy and carbohydrate linkage analysis (Table 1). QS-7 has a smaller molecular weight than QS-21, but is more heavily glycosylated. The main differences in the carbohydrate composition of QS-7 compared to QS-21 were the presence of a terminal glucose, linkage at the 3-position of rhamnose, linkage at the 3-position of fucose, an additional terminal rhamnose, and the absence of arabinose. Mild alkaline hydrolysis is known to convert esterified saponins into deacylsaponins [9]. Hence, we carried out alkaline hydrolysis of the purified saponins and isolated the deacylsaponins to compare the fatty acyl chain of QS-7 with that of QS-21. The molecular weight of the deacylsaponin of QS-21 is 476 lower and does not have arabinose, consistent with hydrolysis of the fatty acid ester bond at the 4-hydroxyl of fucose (Fig. 1). In contrast, mild alkaline hydrolysis of QS-7 to the deacylsaponin does not remove any monosaccharide residues and reduces the molecular weight only by 41. This suggests that QS-7 may be acylated with an acetic acid residue. Both QS-7 and QS-21 were further hydrolysed to the identical prosapogenin (data not shown). A structure of QS-7 that is consistent with the data is shown in Figure 1 and is compared to the known structure of QS-21 [10].

Table 1: Comparison of QS-7 and QS-21.

Saponin	m/z [M+Na] ⁺		Monosaccharide linkage analysis	
	Intact saponin	Deacyl-saponin	Intact saponin	Deacylsaponin
QS-7	1886	1845	t-apiose, t-rhamnose, 3,4-rhamnose, 2,3-fucose, t-xylose, 3-xylose, t-galactose, t-glucose, 2,3-glucuronic acid	Identical to intact saponin
QS-21	2012	1536	t-apiose, 4-rhamnose, 2-fucose, t-xylose, 3-xylose, t-galactose, 2,3-glucuronic acid, t-arabinose	Identical to intact saponin except for loss of arabinose

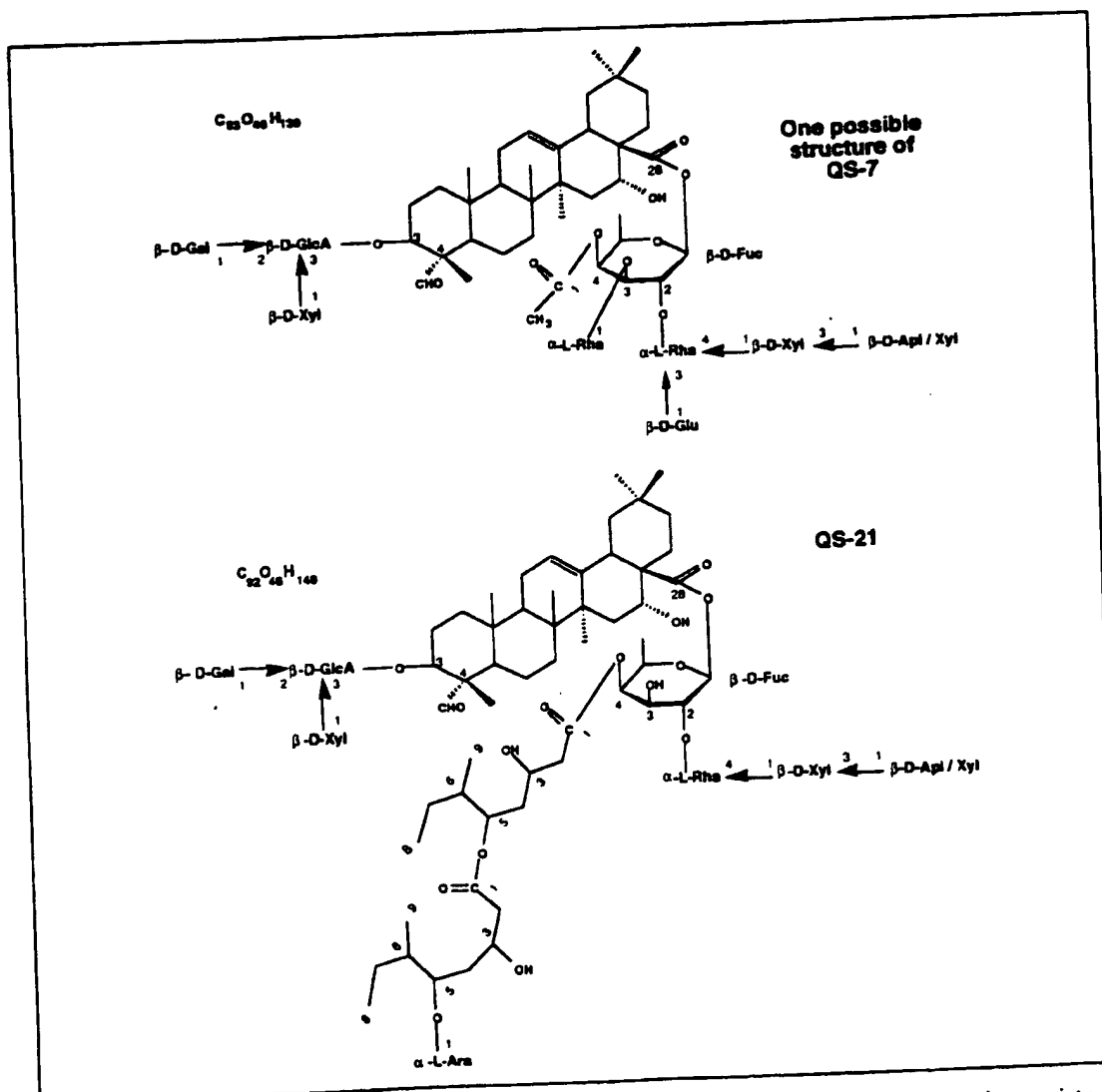


Fig. 1: Saponin structure. The structure shown for QS-7 is one possible structure that is consistent with the FAB-MS and carbohydrate analysis. Structure of QS-21 was determined in Jacobsen et al [10].

Adjuvant activity

QS-7 was evaluated as an adjuvant for inducing cytotoxic T lymphocytes to subunit antigens. The antigen ovalbumin (OVA) was chosen as one model because QS-21 is known to induce high CTL responses to OVA [3]. E.G7-OVA cells, which are EL4 mouse T lymphoma cells transfected with the OVA gene [11], were used as target cells. Figure 2 shows that splenocytes from C57BL/6 mice immunized three times with OVA and QS-7 have a strong antigen-specific cytolytic activity. However, a comparatively high dose of QS-7 (40 μ g and above) was required to stimulate a CTL response similar to that produced by 5 μ g QS-21.

The capacity of QS-7 to stimulate the induction of cytotoxic T lymphocytes was also studied in vaccines consisting of a recombinant glycoprotein, HIV-1_{11B} gp120 (Fig. 3). Balb/c mice were immunized subcutaneously three times with gp120 and various doses of QS-7 or QS-21. The CTL response to HIV-1_{11B} gp120 was measured as a splenocyte-mediated lysis of P815 cells coated with P18 peptide, the predominant CTL epitope in gp120. In contrast to the high doses of QS-7 required to induce CTL with OVA, lower doses of QS-7 stimulated significant CTL to gp120. QS-7 doses of 5 μ g or higher induced CTL responses above background. However, the dose dependence of the response was not clear because the 20 μ g dose of QS-7 induced lower CTL response than the 10 μ g dose of QS-7. This suggests that these doses are below the minimum dose required for optimal priming for p18-specific CTL.

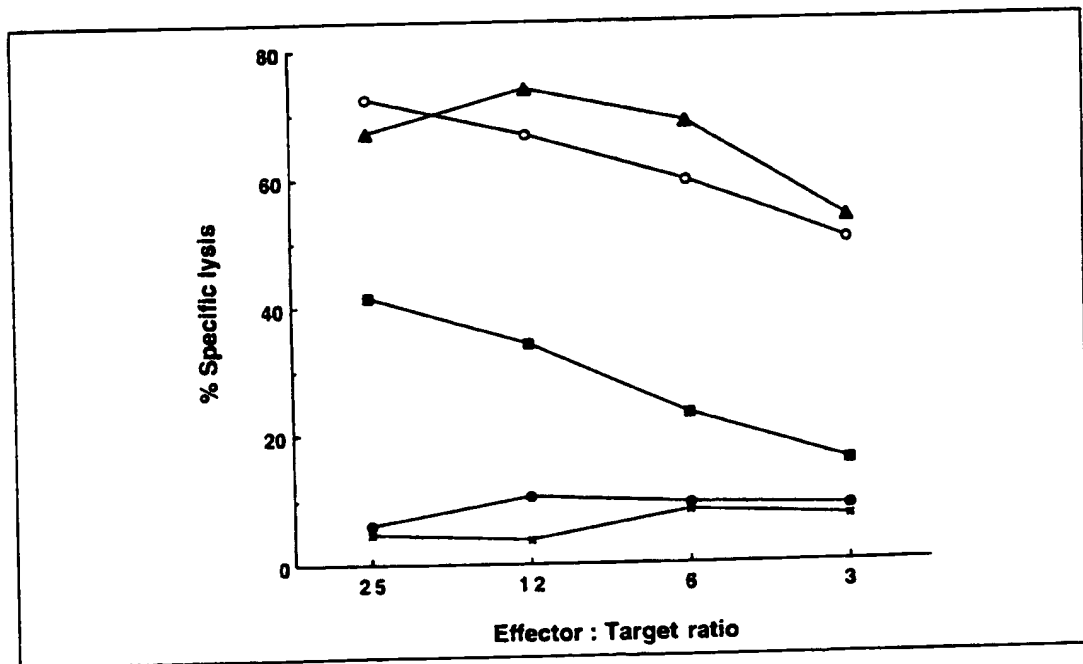


Fig. 2: Cytotoxic T lymphocyte activity of QS-21 and QS-7 with OVA antigen. C57BL/6 mice (5 per group) were immunized subcutaneously at days 0, 14, and 28 with 25 μ g OVA and different doses of QS-7 or QS-21: no adjuvant (x), 5 μ g QS-7 (●), 40 μ g QS-7 (■), 60 μ g QS-7 (▲), and 5 μ g QS-21 (O). Splenocytes were harvested at day 42, stimulated in vitro for six days with mitomycin C-treated E.G7-OVA cells, and used as effector cells in the CTL assay. Lysis was determined by a standard 51 Cr release assay on E.G7-OVA cells (EL4 cells transfected with OVA gene and which express OVA₂₅₇₋₂₆₄ on class I MHC). The background lysis of EL4 cells was subtracted.

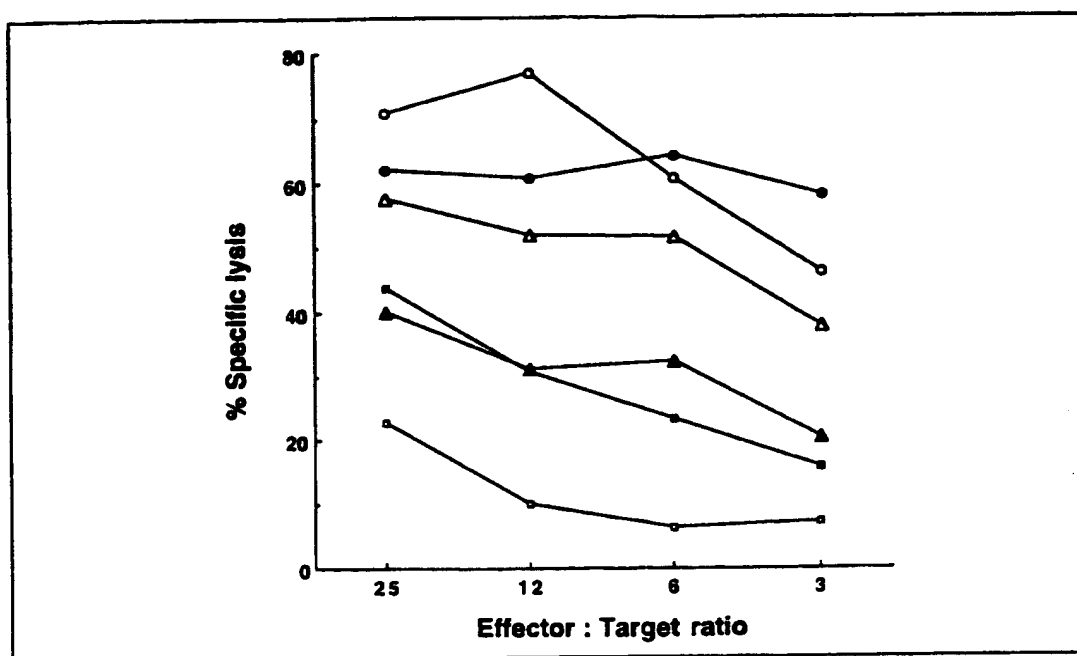


Fig. 3: Cytotoxic T lymphocyte activity of QS-21 and QS-7 with HIV-1 gp120. Balb/c mice (5 per group) were immunized subcutaneously at days 0, 14, and 28 with 25 µg gp120 and different doses of QS-7 or QS-21: no adjuvant (□), 5 µg QS-7 (■), 10 µg QS-7 (△), 20 µg QS-7 (▲), 40 µg QS-7 (●), and 10 µg QS-21 (O). Splenocytes were harvested at day 42, stimulated in vitro for six days with HIV-1 gp120 peptide 18 (predominant CTL epitope), and used as effector cells in the CTL assay. Lysis was determined by a standard ^{51}Cr release assay on P815 cells coated with the peptide. The background lysis of P815 cells was subtracted.

Lytic activity

One of the properties of most esterified bisdesmodic triterpene saponins is a haemolytic activity. QS-21, at concentrations of approximately 7 µg/ml (3.5 µM), is haemolytic to sheep red blood cells in an in vitro assay [2]. Haemolytic activity is reported to be especially high with esterified saponins. Although QS-7 is an esterified saponin, it has a considerably shorter acyl chain than QS-21. The concentration of QS-7 required for 50% haemolysis was 237 µM.

DISCUSSION

Although many adjuvants are effective in increasing humoral immune responses, fewer adjuvants will improve cell-mediated immune responses, particularly to soluble antigens. Soluble, nonparticulate antigens are not typically processed by the class I major histocompatibility (MHC) antigen pathway that leads to CD8⁺ CTL responses to antigens synthesized in the cytoplasm («endogenous» antigens), but are instead «exogenous» antigens which are internalized by endocytosis and processed by the class II MHC pathway that leads to antibody responses. Hence, CTL responses are difficult to raise against soluble antigens. However, certain adjuvant and antigen delivery strategies have been shown to modify the

response for soluble antigens from a Class II MHC response to a Class I MHC response. These include antigen delivered in acid-sensitive liposomes [12], lipopeptides [13], and ISCOMs [14]. These may act as lipophilic carriers for directing antigen through the endosomal membrane into the cytosol.

QS-21 is another adjuvant that is known to induce strong CD8⁺ CTL response to subunit antigens. Although it does not form a complex with soluble, hydrophilic antigens such as OVA, it does interact with cell membranes due to its amphipathic structure. It is one of the more hydrophobic saponins in *Q. saponaria* extracts and is known to be taken up by splenocytes and lymph node cells and will bind to liposomes of phosphatidylcholine/cholesterol (unpublished data). Hence, it is possible that it intercalates into the membranes of APC and enables escape of exogenous antigen into the cytoplasm. Deacylated QS-21 was previously shown to be inactive as an adjuvant for CTL responses [15]. This suggests that esterification may be important for this activity. However, the length of the fatty acid chain may be less important. QS-7, which appears to be esterified with an acetic acid, can clearly stimulate CTL responses. For example, it is an excellent inducer of CTL responses to HIV gp120. With OVA, a several fold higher dose of QS-7 is required to induce a CTL response comparable to that of QS-21. The trend with OVA is that the saponin with higher lytic activity (QS-21) is a stronger inducer of CTL. However, the differences in optimum dose required for induction of CTL to OVA is considerably lower than predicted by the substantial differences in the haemolytic activity between QS-21 and QS-7 (70 fold difference in haemolytic titre). It is possible that there is a stronger correlation between CTL activity and effect of these saponins on membranes of APC than there is with haemolytic activity.

CONCLUSION

QS-7, a hydrophilic saponin from *Quillaja saponaria*, can stimulate a cell-mediated immune response to HIV-1 gp120 and OVA. Preliminary structural studies suggest that this saponin is a highly glycosylated bisdesmoside quillaic acid saponin, acylated with an acetic acid.

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Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function

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QS-21, a purified Quillaja saponaria saponin immunologic adjuvant, contains two functional groups that we hypothesized to be involved in the adjuvant mechanism of action through charge or Schiff base interaction with a cellular target. Derivatives, prepared by modification of these sites, were prepared and tested for their ability to augment the immunogenicity of the antigen ovalbumin (OVA) in C57BL/6 mice. QS-21 derivatives that were modified at the carboxyl group on an anionic sugar, glucuronic acid, retained adjuvant activity for antibody stimulation, inducing relative increases in antibody titers similar to those induced by QS-21, although the minimum adjuvant dose required for this stimulation was increased several fold relative to the dose of unmodified QS-21. One of these derivatives also retained significant activity for induction of OVA-specific cytotoxic T-lymphocytes. In contrast, QS-21 derivatives modified at an aldehyde on the triterpene did not show adjuvant activity for antibody stimulation or for induction of cytotoxic T-lymphocytes, suggesting that this functional group may be involved in the adjuvant mechanism.

Keywords: QS-21; adjuvant; *Quillaja saponaria*; saponin; structure/function

Extracts of the bark of the South American tree *Quillaja saponaria* Molina contain a heterogeneous saponin fraction with potent adjuvant activity^{1,2}. These saponins have been purified to near homogeneity by HPLC and characterized for adjuvant activity³. Several were shown to stimulate high antigen-specific antibody titers in mice^{3,4}. One of these saponins, QS-21, was also shown to induce class I MHC-restricted cytotoxic T-lymphocyte (CTL) responses in mice when used with subunit antigens such as ovalbumin and recombinant HIV-1 envelope antigens^{5,6} and to increase antibody titers to T-independent polysaccharide antigens⁷. QS-21 has also been tested as an adjuvant in a Phase I melanoma vaccine clinical trial and was noted to augment antigen-specific IgG titers⁸, making it of particular interest as a vaccine adjuvant. However, relatively little is known of the minimum critical structure of QS-21 required for these adjuvant functions. This study addresses the relationship of QS-21 structure to its adjuvant function via analysis of two functional groups.

QS-21 is a highly complex triterpene glycoside (*Figure 1*), with branched sugar chains at carbon 3 and carbon 28 on the triterpene quillaic acid⁴. A correlation between the presence of branched sugar chains at these positions and adjuvant activity of naturally occurring saponins was noted⁹. The glycoside on carbon 3 contains an anionic sugar residue, glucuronic acid, which imparts an overall negative charge to the QS-21 molecule at physiological pH. There is also an aldehyde on carbon 4 of the triterpene. In addition, QS-21 contains a fatty acid (3,5-dihydroxy-6-methyl-octanoic acid) linked through an ester bond to the 3-hydroxyl or 4-hydroxyl of fucose (N. Jacobsen, personal communication). An identical fatty acid is linked in ester bond to the 5-hydroxyl of the first fatty acid; the 5-hydroxyl of the second fatty acid is glycosylated with a single sugar (arabinose).

Relatively little is known of the minimum critical structure of QS-21 required for adjuvant function. There are several adjuvant active saponins that have been isolated from *Quillaja saponaria* Molina³. These include the saponins QS-7, QS-17, QS-18, and QS-21 which are the predominant saponins in the bark and which as an aggregate represent approximately half of the saponins present in *Quillaja saponaria* bark. Structural comparison suggests that the known adjuvant active saponins have the triterpene backbone (quillaic acid) and some

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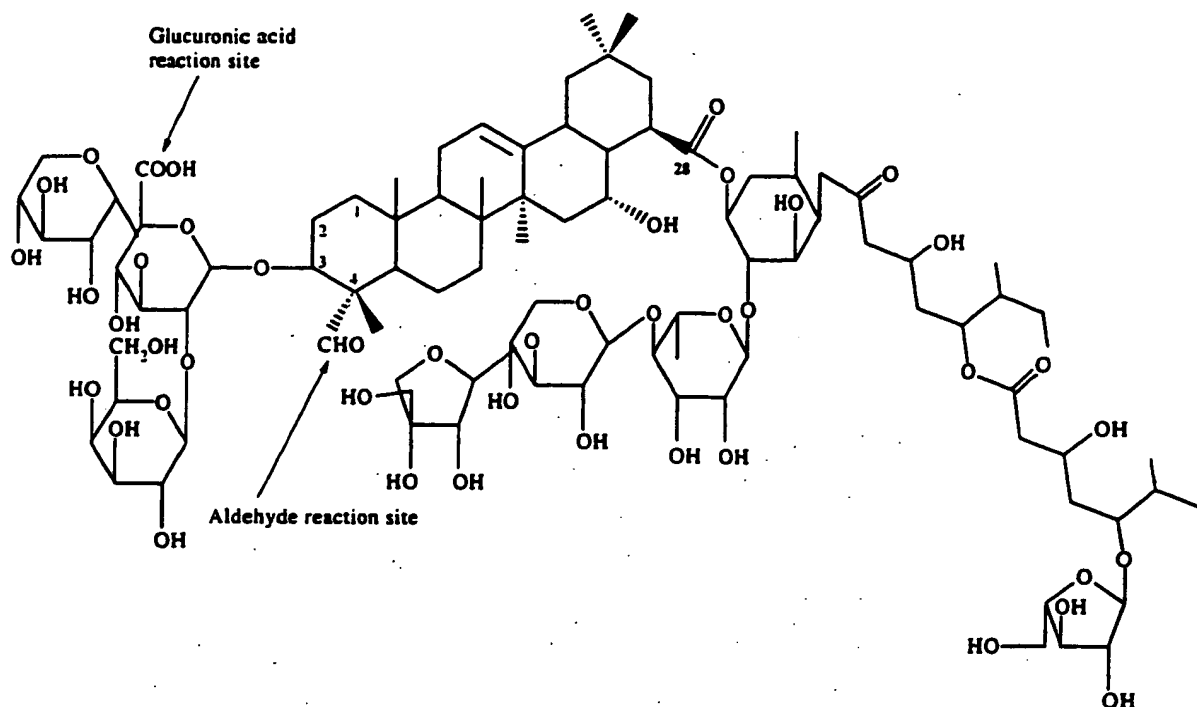


Figure 1 Structure of QS-21. The two functional groups selected for modification are shown

carbohydrate residues in common. Two structural features held in common by all adjuvant active saponins from *Quillaja saponaria* Molina are the 2,3 glucuronic acid^{3,4,10} and the quillaic acid backbone, including the aldehyde at carbon 4¹¹. We postulated that these functional groups were involved in the QS-21 adjuvant mechanism, the glucuronic acid through charge interaction and the aldehyde via Schiff base formation with a cellular target. Hence, these two functional groups (glucuronic acid carboxyl group and triterpene aldehyde) were modified by conjugation to small blocking groups; the resulting derivatives were tested as adjuvants in an effort to evaluate the importance of these functional groups or nearby regions to adjuvant function.

MATERIALS AND METHODS

Materials

QS-21 was purified from an aqueous extract of *Quillaja saponaria* bark by adsorption chromatography and reversed-phase HPLC³. N-hydroxysulfosuccinimide (S-NHS) and N,N'-dicyclohexylcarbodiimide (DCC) were purchased from Pierce Chemical Co. (Rockford, IL). Sodium cyanoborohydride, anhydrous dimethylformamide (DMF), anhydrous dimethylsulfoxide (DMSO), and 8-anilino-1-naphthalene-sulfonic acid (ANS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ovalbumin (OVA), Grade VI, was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of derivatives modified at glucuronic acid carboxyl

Conjugates (1:1 mol:mol) of QS-21 and the small molecules ethylamine, glycine, and ethylenediamine were prepared. In order to react the glucuronic acid carboxyl group of QS-21 with free amino groups on the molecules used as blocking agents, an active ester

derivative of QS-21 was prepared using sulfo-NHS¹². A twofold molar excess of S-NHS and a threefold molar excess of DCC were added to 50 mM QS-21 in anhydrous DMF; this reaction mixture was incubated with stirring overnight at room temperature to generate the S-NHS active ester derivative of QS-21 (QS-21/S-NHS ester). The reaction mixture was then chilled on ice and filtered to remove dicyclohexylurea. The active ester derivative of QS-21 was then precipitated by addition of ethylacetate (final ratio=6/1 ethylacetate/DMF, v/v), purified by repeated washes with ethylacetate, collected by centrifugation (15 min at 1000g), and dried by vacuum desiccation overnight.

In order to conjugate the small molecules glycine, ethylamine, and ethylenediamine through their free amino groups to the glucuronic acid carboxyl of QS-21, a modification of the method of Anjaneyulu and Staros¹³ was employed. A 100-fold molar excess of glycine, ethylamine, or ethylenediamine (1.0 M in 0.1 M sodium phosphate, pH 7.0) was added to solid QS-21/S-NHS active ester. The reaction mixture was stirred at room temperature for 1 h. The resulting conjugates were purified on a Vydac C4 column (1.0 cm I.D. × 25 cm length, 5 μm particle size, 300 angstrom pore size) using a Waters 600E HPLC system and detection at 214 nm (LambdaMax Model 481 Variable Wavelength Detector). A linear water/acetonitrile gradient in 0.15% trifluoroacetic acid was used. Pooled fractions containing the predominant reaction product were lyophilized to dryness.

Preparation of derivatives modified at triterpene aldehyde

To conjugate glycine, ethylamine, and ethylenediamine through their free amino groups to the triterpene aldehyde on QS-21 (Figure 1), a 50-fold molar excess of these compounds in 0.1 M sodium phosphate, pH 6.0

was added to QS-21 (12 mM) in 0.1 M sodium phosphate (pH 6.0)/methanol (50/50, v/v) and incubated with stirring overnight at room temperature to induce Schiff base formation. These adducts were stabilized by the use of sodium cyanoborohydride as a selective reducing agent¹⁴. Sodium cyanoborohydride (from a 0.1 M stock solution in methanol) was added to a final ratio of 4/1 (mol/mol) over QS-21. The reaction mixture was stirred overnight. The predominant reaction product was purified by HPLC as described above.

Characterization of QS-21 derivatives

Derivatives were characterized for purity and retention time relative to QS-21 by reversed-phase HPLC on C18 (3 μ m particle size, 120 angstrom pore size, 4.6 mm I.D. \times 15.0 cm length (YMC Inc., Wilmington, N.C.)) using a linear gradient of 80% solvent A/20% solvent B to 40% solvent A/60% solvent B over 20 min at a 1 ml min⁻¹ flow rate. Solvent A was 0.1% H₃PO₄ in water and solvent B was 0.1% H₃PO₄ in acetonitrile. Detection was by UV absorbance at 205 nm. Relative retention time was determined from the ratio of k' derivative/ k' QS-21 where k' = (peak retention time - void retention time)/(void retention time). Molecular weights were determined by fast atom bombardment-mass spectrometry (M-Scan Corp., Westchester, PA) to confirm that these derivatives were 1:1 covalent conjugates. ¹H-NMR on samples in deuterated dimethylsulfoxide was carried out by Spectral Data Services (Champaign, IL). Modification of the triterpene aldehyde in compounds (5)-(7) was confirmed by elimination of the aldehyde proton resonance (singlet with chemical shift = 9.47 in unmodified QS-21). The aldehyde proton resonance was present in compound (3) whereas a proton singlet at 8.1 ppm (assigned as the proton resonance on the amide bond nitrogen) appeared. Compounds (4) and (5) were not assayed by NMR.

Immunizations

C57BL/6 mice (female, 8-10 weeks of age) were used for all immunizations. Mice were immunized subcutaneously with 0.2 ml of 25 μ g OVA and varying amounts of QS-21 derivatives with either two or three immunizations spaced 2-3 weeks apart. Sera were collected one week after the second immunization or two weeks after the third immunization for analysis by EIA. Splenic mononuclear cells for use as the source of effector cells in the CTL assay were collected two weeks after the last of three immunizations.

Immunological assays

The EIA was done using OVA-coated Immulon IV plates (Dynatech Laboratories, Chantilly, VA). Plates were coated with OVA by overcoating wells with 100 μ l per well of 10 μ g ml⁻¹ OVA in PBS and incubation at 4°C overnight. Plates were emptied and were then incubated for 1 h at ambient temperature with 150 μ l well⁻¹ of 10% normal goat serum (Gibco Laboratories, Grand Island, NY) in PBS. Plates were washed three times with 0.05% Tween-20 in water. Serial dilutions of sera in 10% normal goat serum in PBS (1/10 dilutions) were prepared and incubated on the plate for 1 h at room

temperature. Plates were then washed three times with 0.05% Tween-20 in water. For measurement of total IgG, a total volume of 100 μ l goat anti-mouse IgG-horseradish peroxidase conjugate (BioRad, Richmond, VA), diluted 1/12 500 in 10% normal goat serum in PBS, was incubated on the plate for 1 h at room temperature. For measurement of IgG1, IgG2_b, or IgG2_a, a volume of 100 μ l goat-antimouse IgG1, IgG2_b, or IgG2_a (conjugated to alkaline phosphatase, Southern Biotechnology, Birmingham, AL, diluted 1/250 in 10% normal goat serum/PBS) was added to each well and incubated at 4°C overnight. Plates were washed five times with 0.05% Tween-20 in water followed by one wash with water. Color development was with tetramethylbenzidine substrate for total IgG and with *p*-nitrophenylphosphate for IgG1, IgG2_b, and IgG2_a.

CTL activity was assayed using splenic mononuclear cells as the source of CTL. The precursor CTL in splenic mononuclear cell preparations were induced to mature *in vitro* by culture with mitomycin C-treated E.G7-OVA cells at a 20:1 (responder:stimulator) ratio or with denatured ovalbumin. For a negative antigen control, splenic mononuclear cells were cultured with medium (supplemented RPMI 1640). Culture in presence or absence of antigen was carried out using supplemented RPMI 1640 medium at 37°C in a 2 ml volume with 1×10^6 cells per ml in culture tubes. Cells were recovered after 144 h of culture and used in the CTL assay. The CTL activity was measured using both EL4 cells and E.G7-OVA (EL4 cells transfected with cDNA coding for OVA¹⁵) as targets. Cytotoxicity was measured after 4 h of incubation of CTL with 10^4 ⁵¹Cr-labeled target cells per well, using effector/target (E/T) ratios of 25:1 to 3:1. The percent of specific ⁵¹Cr release was calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ where maximum release was measured after lysis of target cells with 1% NP-40 and spontaneous release was measured after incubation of target cells with medium.

Hemolysis and critical micellar concentration assays

Hemolytic activity of QS-21 and derivatives were measured in an *in vitro* assay on sheep red blood cells. Five milliliters of sheep red blood cells in Alsever's solution (Biowhittaker, Walkersville, Maryland) were spun at 900g for 5 min. The pellet was resuspended in 5 ml PBS. This process was repeated twice. The final pellet was resuspended in 3 ml PBS. One hundred microliters of serial 1:2 dilutions of QS-21 or derivatives in PBS were added to individual wells of Falcon flexible round bottom 96 well assay plates (Becton Dickinson, Oxnard, CA). Twenty-five microliters of the washed red blood cell suspension were added to each well, mixed with the saponin solution, and incubated at room temperature for 30 min. The round bottom plate was then centrifuged at 1000g for 5 min. Fifty microliters of each of the supernatants were transferred to wells in a flat bottom microtiter plate for determination of the absorbance at 570 nm.

Critical micellar concentration (cmc) of QS-21 and derivatives was determined by a fluorescent dye binding assay as described previously^{16,17}. The fluorescent probe ANS, 11 μ M in phosphate-buffered saline, was mixed with different concentrations of QS-21 or derivative.

Immediately after mixing, fluorescence emission at 490 nm with excitation at 370 nm was determined. The fluorescence emission was plotted versus QS-21 or derivative concentration. Biphasic curves were obtained with low fluorescence emission below the cmc and significant increase in fluorescence emission above the cmc due to intercalation of ANS into the micelle. Best fit lines were determined for the biphasic curve; the cmc was defined as the QS-21 or derivative concentration corresponding to the intersection of the best fit lines.

RESULTS

Modification of QS-21

In order to evaluate the importance of the glucuronic acid and triterpene aldehyde to the adjuvant activity of QS-21, derivatives consisting of 1:1 conjugates of QS-21 linked to the small blocking groups glycine, ethylamine, and ethylenediamine at these functional groups were prepared. The size, charge, and hydrophobicity of these derivatives compared to QS-21 are summarized in Table 1. Although the size increase to QS-21 due to conjugation of these blocking groups was minimal, all of the conjugations were expected to sterically hinder or block any direct interactions at the modified sites. Some of these modifications also modified QS-21 charge, enabling an evaluation of whether the anionic carboxyl group on QS-21 was involved in a charge interaction as part of the adjuvant mechanism. In compound (3), the glucuronic acid was blocked with a neutral group (ethylamine), eliminating the charge at physiological pH. In compound (4), conjugation of the acid to ethylenediamine resulted in a conjugate with a free amino group, imparting a cationic charge. Although modifications of the aldehyde did not affect the anionic group on the glucuronic acid carboxyl, this modification did affect the overall charge of the QS-21 molecule. Compound (6) is zwitterionic, with negative charges on the glucuronic acid and a positive charge on the secondary amine formed on the aldehyde, whereas compound (7) is positively charged overall. Hydrophobicity changes (assessed by relative retention time to QS-21 on reversed-phase HPLC) were determined to be minimal and were within the range encompassed by naturally occurring adjuvant-active saponins such as QS-7 (relative retention time 0.72).

Antibody stimulation

All derivatives were tested for activity in stimulating antigen-specific antibody to OVA in C57BL/6 mice. Mice received three immunizations with OVA and 10 µg of QS-21 (1) or derivatives (2)–(4) (modified at the glucuronic acid carboxyl group) or (5)–(7) (modified at the aldehyde at triterpene carbon 4). Anti-OVA IgG1, IgG2_b, and IgG1 titers were determined by EIA (Figure 2). Unmodified QS-21 induced significant increases in IgG1, IgG2_b, and IgG2_a. Derivatives (2) and (3) also induced significant increases in antibody of all three isotypes although to a slightly lesser extent than QS-21. No adjuvant effect was noted for derivative (4) except for a 10-fold increase in IgG2_b. The antibody profile induced by OVA formulations adjuvanted with derivatives (5)–(7) was similar to that induced by the OVA/

Table 1 Characterization of QS-21 derivatives

Derivative	Blocking group	Reaction site	Theoretical molecular formula (formula weight) ^a	m/z of pseudo-molecular ions (relative intensity) ^b	Assignment of FAB-MS ion peaks	Relative retention time	Theoretical charge at physiological pH
(1)	None	None	C ₆₂ O ₁₆ H ₁₄₉ (M = 1988.9)	2012 (100%)	(M+Na) ⁺	1.00	-
(2)	Glycine	Glucuronic acid carboxyl	C ₆₄ O ₁₇ N ₁ H ₁₅₁ (M = 2045.9)	2085 (100%)	(M+Na) ⁺	0.97	-
(3)	Ethylamine	Glucuronic acid carboxyl	C ₆₄ O ₁₆ N ₂ H ₁₅₃ (M = 2016.0)	2039 (100%)	(M+K) ⁺ (M+Na) ⁺	1.08	0
(4)	Ethylenediamine	Glucuronic acid carboxyl	C ₆₄ O ₁₅ N ₃ H ₁₅₄ (M = 2031.0)	2054 (100%)	(M+Na) ⁺	0.80	+
(5)	Glycine	Aldehyde	C ₆₄ O ₁₇ N ₁ H ₁₅₃ (M = 2048.0)	2071 (100%)	(M+Na) ⁺	0.83	-
(6)	Ethylamine	Aldehyde	C ₆₄ O ₁₆ N ₂ H ₁₅₅ (M = 2018.0)	2019 (100%)	(M+H) ⁺	0.90	0
(7)	Ethylenediamine	Aldehyde	C ₆₄ O ₁₅ N ₃ H ₁₅₆ (M = 2033.0)	2034 (100%)	(M+H) ⁺	0.78	+

^aTheoretical formula weight was calculated from the exact mass of the commonest isotope of each element. ^bThe m/z values for the most predominant peaks noted in fast atom bombardment-mass spectra are reported. The relative intensities of these peaks (expressed as % of the intensity of the most predominant peak) are reported in parentheses. The most predominant peak for QS-21 and most derivatives was the sodium adduct (M+Na)⁺.

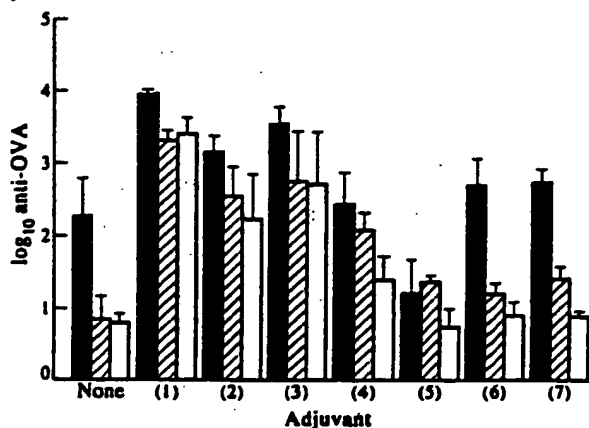


Figure 2 Antigen-specific antibody stimulation by QS-21 derivatives. C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10 and 12 weeks of age with test formulations containing 25 μ g OVA adjuvanted with 10 μ g QS-21 or QS-21 derivative. A control group immunized with OVA in saline was included. Serum were collected one week after the third immunization and analyzed for anti-OVA of the IgG subclasses IgG1 (solid bars), IgG2_a (cross-hatched bars), and IgG2_b (stippled bars). Data are expressed as the mean and 1 standard error of the log₁₀ titer of the five mice in each group

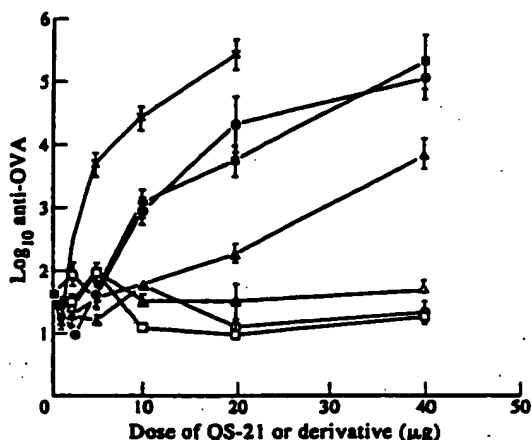


Figure 3 Effect of QS-21 derivative dose on antibody stimulation. C57BL/6 mice (10 per group) were immunized subcutaneously with 25 μ g OVA and the indicated dose of QS-21 or QS-21 derivative in a total volume of 0.2 ml saline at 8 and 10 weeks of age. Sera were collected 1 week after the second immunization and an equivolume pool was made from each of the mice in a group; these pools were analyzed in quadruplicate by EIA on plates coated with OVA. Data are reported as the mean and 1 standard error of log₁₀ titer. Test adjuvant: (1), X; (2), ●; (3), ■; (4), ▲; (5), ○; (6), □; (7), △

saline formulation, suggesting that these derivatives were inactive as adjuvants. The total IgG titer (not shown) also indicated the same trend.

The anti-OVA total IgG response to different doses of derivatives were compared in Figure 3. Mice were immunized subcutaneously with test vaccines containing 25 μ g OVA and doses of QS-21 or derivative ranging between 2.5 and 40 μ g. Serum was collected at 1 week after a second immunization and tested for total IgG to OVA. QS-21 stimulated anti-OVA IgG at doses between 5 and 10 μ g, with some partial effect observed at 2.5 μ g. A higher dose was required for QS-21 derivatives which were modified at the glucuronic acid carboxyl ((2)–(4)). However, despite the increase in minimum effective dose, all derivatives modified at the carboxyl retained

the function of antibody stimulation. In addition to total IgG, antigen-specific IgG1, IgG2_b, and IgG2_a were measured for these derivatives and were observed to increase according to the same dose response curves as for the total IgG (data not shown). In contrast, none of the derivatives prepared by conjugation to the triterpene aldehyde ((5)–(7)) stimulated any increase in antibody titer despite doses of up to 40 μ g (approximately 16-fold higher than the lowest dose of QS-21 (2.5 μ g) that stimulates any significant increase in titer). Hence, modification of the triterpene aldehyde of QS-21 effectively eliminated its property of antibody stimulation in this dose range, although activity at a higher dose could not be ruled out.

Class I-restricted cytotoxic T-lymphocyte stimulation

One of the more unique properties of QS-21 adjuvant is its activity for stimulation of MHC class I-restricted CTL in response to subunit vaccines. Hence, the derivatives were tested for stimulation of CTL to determine whether modification affects this property. The results are summarized in Figure 4. An OVA-transfected syngeneic cell (E.G7-OVA) was used as target. Splenocytes (from mice receiving 3 immunizations with test vaccines) were stimulated by mitomycin-C treated E.G7-OVA to induce CTL maturation; these splenocytes were then used as effector cells in the lysis assay (panel A). The specific killing induced by effector cells from mice receiving derivatives as adjuvants were compared to effectors from mice receiving QS-21 and was used as a measure of precursor CTL induced by these adjuvants. Additionally, denatured OVA was tested as an antigen stimulus to determine whether the derivatives induced an APC population with capacity for antigen processing (panel B).

Derivative (3) (prepared by modification of the glucuronic acid carboxyl with ethylamine) was as effective as QS-21 in stimulating precursor CTL that could be stimulated to mature by stimulation with mitomycin-C treated E.G7-OVA cells. Derivative (3) also induced precursor CTL that could be expanded by denatured OVA, although to a lesser extent than QS-21. Derivatives (2) and (4) (prepared by modification of the glucuronic acid carboxyl with glycine and ethylenediamine, respectively) also stimulated a CTL response. However, this response was lower than that induced by QS-21 and was observed only by use of processed antigen (mitomycin C-treated E.G7-OVA cells) as an antigen stimulus. Doses higher than 10 μ g were not tested, so the possibility that these derivatives would induce a stronger lytic response at doses yielding maximum response in antibody stimulation cannot be ruled out. Derivatives (5)–(7) (prepared by modification of the triterpene aldehyde) were not active or poorly active in CTL induction, invoking responses that were similar to that induced by a nonadjuvanted OVA vaccine.

Effect of modification on detergent properties of QS-21

QS-21 and other saponins from *Quillaja saponaria* associate in micelles due to their amphipathic structure. As a measure of the propensity of these molecules to form micelles, the critical micellar concentration was determined (Table 2). The critical micellar concentration

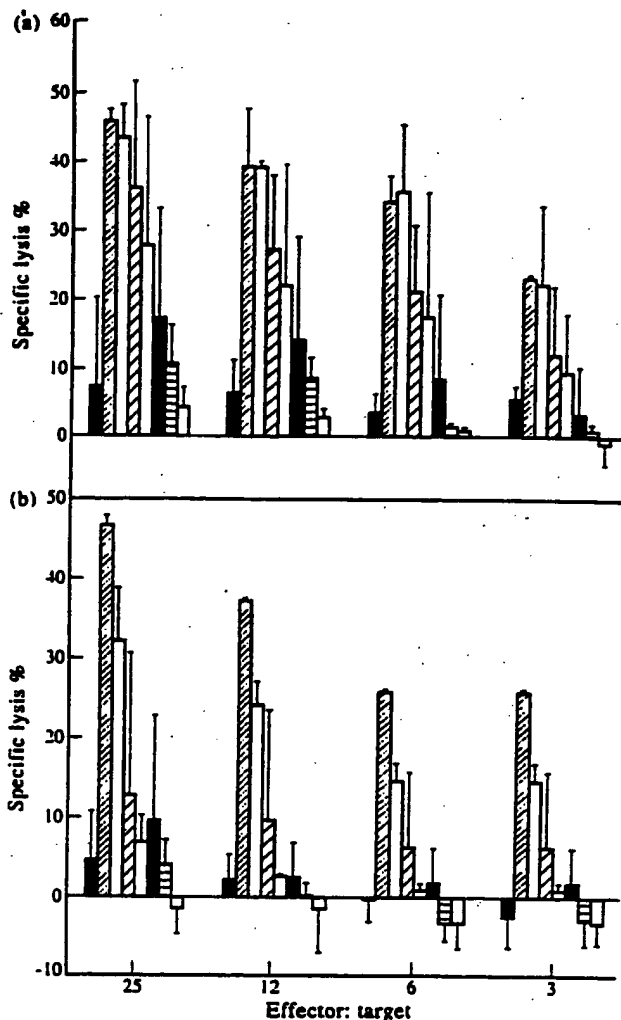


Figure 4 Cytotoxic T-lymphocyte stimulation by QS-21 derivatives. C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10, and 12 weeks of age with test formulations containing 25 μ g OVA adjuvanted with 10 μ g QS-21 or QS-21 derivative. CTL activity was measured using E.G7-OVA cells and EL-4 cells as targets and splenic mononuclear cells from immunized animals as CTL effector cells. Splenic mononuclear cells were collected from 2 to 4 weeks after the last immunization. Maturation of precursor CTL to functional effector cells *in vitro* was carried out by specific antigen stimulation using mitomycin C-treated E.G7-OVA cells (panel A) and denatured OVA (panel B). Data are expressed as mean % specific lysis \pm 1 standard deviation from two separate assays of pooled splenocytes from 2 or 3 mice after subtraction of background lysis of EL4 cells. Test adjuvant: None ■; QS-21, (1), ▨; (3), ▩; (4), ▤; (2), □; (6), □; (7), ▦; (5), □

was minimally affected by modification of the glucuronic acid, with these derivatives having lower cmc values than the intact QS-21. This was consistent with the site of modification, which is in one of the hydrophilic domains of the QS-21 molecule. In contrast, the cmc values of the derivatives modified at the aldehyde were substantially higher than that of QS-21, ranging from 2.3-fold higher for the cationic ethylenediamine derivative to almost sixfold higher for the anionic glycine derivative, consistent with modification of QS-21 in one of the two hydrophobic domains expected to contribute to association.

The lysis of red blood cells in an *in vitro* assay was used as a second measure of detergent activity. The relative hemolytic activity of the derivatives was measured and compared to that of unmodified QS-21

Table 2 Detergent properties of QS-21 derivatives

Derivative	Critical micellar concentration (μ M) ^a	Concentration inducing 50% hemolysis (μ M) ^b
QS-21 (1)	26.6 \pm 4.9	4
(2)	22.0 \pm 7.7	16
(3)	16.0 \pm 2.2	7
(4)	13.3 \pm 8.2	17
(5)	147 \pm 2.1	>244 ^c
(6)	89.6 \pm 0.9	>248
(7)	61.0 \pm 8.2	>248

^aThe critical micellar concentration was determined in phosphate-buffered saline, pH 7.0, as described in Materials and Methods. Data is expressed as mean \pm 1 standard deviation in assay of two separate preparations of each derivative. ^bThe concentration inducing 50% hemolysis of sheep erythrocytes in an *in vitro* assay was determined as described in Materials and Methods. ^cHighest concentration tested

(Table 2). Modifications made at the glucuronic acid did not substantially affect the hemolytic activity. However, modifications at the triterpene aldehyde eliminated hemolytic activity up to the highest concentration tested (244–248 μ M). The results were consistent with the results from the critical micellar concentration determination, indicating that the lysis of cellular membranes was affected by modifications that increased the cmc. For QS-21 and those derivatives that retained hemolytic activity, minimum hemolytic concentrations were lower than the cmc, indicating that the monomeric form of QS-21 and derivatives is the form which associates with the erythrocyte membrane.

DISCUSSION

All derivatives were tested for adjuvant activity by determining their effects on both antibody response and cellular mediated response to determine whether these activities were affected equally or would be affected in an unequal fashion. Augmentation of antibody but not CTL or the converse would suggest that different QS-21 functional groups or regions are involved in these separate activities. However, derivatives prepared by modification at the glucuronic acid carboxyl were active for stimulation of both antibody and CTL; derivatives prepared by modification of the triterpene aldehyde were inactive for both responses. This does not rule out the possibility that these activities can be mapped to separate regions that we have not yet examined. For example, diphosphoryllipid A has both adjuvant and toxic properties, but the derivative monophosphoryllipid A retains adjuvant activity, but exhibits significantly lower toxicity than the parent molecule¹⁸.

Some moderate association between detergent activity and adjuvant activity was noted in this study. This was most evident in the three derivatives which were modified at the triterpene aldehyde. This modification resulted in substantial increases in both the critical micellar concentration and in concentrations required for hemolytic activities, indicating that the modification of the apolar triterpene interferes with self-association and membrane association. This was correlated with a loss of adjuvant activity. By contrast, modification of glucuronic acid, which is part of the hydrophilic glycoside and is not expected to participate in self-association,

did not substantially affect the detergent properties of this molecule and adjuvant activity was retained at high doses. However, this correlation between loss of hemolytic activity and loss of adjuvant activity is different from our previous observation that a naturally occurring *Quillaja saponaria* saponin, QS-7, is not hemolytic up to $500 \mu\text{g ml}^{-1}$, but is adjuvant active³. Derivative (3) (conjugation of ethylamine to the glucuronic acid) is more hydrophobic than QS-21, has a lower critical micellar concentration than QS-21, and has similar hemolytic activity. However, the minimum effective dose of (3) indicated by the dose response for stimulation of antibody was several fold higher than native QS-21. We have previously observed that QS-21 is active as an adjuvant below the critical micellar concentration¹⁷. Hence, the adjuvant properties of QS-21 are not necessarily associated with its lysis of cell membranes or its properties of self association or membrane association.

Studies with muramyl dipeptide (MDP) derivatives such as MTP-PE suggest that increasing hydrophobicity does not substantially affect antibody stimulation, but improves cell-mediated responses¹⁹. Lipophilicity was also noted to be important for the adjuvant effect of nonionic block copolymer adjuvants²⁰. However, lipophilic derivative (3) of QS-21 was not improved over QS-21 in either minimum dose and levels of antibody stimulation or induction of cell-mediated immune responses, measured in this study as induction of Class I restricted CTL. However, it did induce the highest CTL response of the derivatives, suggesting that an increase in lipophilicity does influence this function. Although most of the derivatives were apparently less hydrophobic than QS-21, being retained less tightly on reversed-phase HPLC, the range of retention times of these derivatives was encompassed by that of naturally occurring, adjuvant-active, polar saponins from *Quillaja saponaria* such as QS-7, QS-17 and QS-18, which stimulate antibody response in mice in the same dose range as QS-21^{3,4}. Hence, we expected that any differences observed in biological function of these derivatives would be primarily due to blocking of the functional groups, with the polarity changes playing a relatively minor role in the differences.

The three derivatives prepared by conjugation of small molecules to the glucuronic acid carboxyl retained substantial adjuvant activity. In addition to stimulating total antigen-specific IgG titer, these derivatives were active in stimulating antigen-specific IgG1, IgG2_b, and IgG2_a, suggesting a stimulation of both Th1 and Th2 cells. The ability to stimulate IgG2_b and IgG2_a antibody has been noted previously for unmodified QS-21^{3,4}, suggesting that modification at the glucuronic acid does not affect the Th1-type response associated with QS-21. Furthermore, these derivatives were active as adjuvants for induction of MHC Class I-restricted precursor CTL. Immunization with antigen/QS-21 has been suggested to set up an activated macrophage population that is highly efficient in the presentation of exogenously provided antigen such as OVA to the Class I MHC pathway²¹. One of the derivatives appeared to retain this property. The effector cells from mice immunized with OVA and derivative (3), modified at the glucuronic acid with ethylamine, were stimulated to produce mature CTL after stimulation with denatured OVA, suggesting that

this derivative retained the ability of the native QS-21 molecule for activation of this APC population for processing and presenting OVA antigen. Hence, derivatives modified at the glucuronic acid carboxyl retained the ability to set up the same type of immunological responses as the native QS-21. This was in spite of modifications that blocked the anionic carboxyl group with a neutral or cationic molecule. Hence, it is unlikely that this functional group is directly involved in adjuvant function. However, the increase in minimum effective dose with these derivatives relative to QS-21 suggests a potential steric hindrance of a site important to activity. It has been proposed that the glucuronic acid on *Quillaja* saponins prevents aggregation of immune-stimulating complexes (ISCOM) containing *Quillaja* saponin²². Because we were able to utilize the QS-21 derivatives as adjuvants in soluble form, we did not attempt to prepare ISCOM with these QS-21 derivatives to determine whether the elimination of the charge affected ISCOM formation.

The glucuronic acid site is a potential site for conjugation directly to antigen. Conjugation of muramyl dipeptide adjuvant directly to either luteinizing hormone-releasing hormone²³ or to coliphage MS-2 viral peptide coupled to a polymeric carrier²⁴ yielded highly immunogenic complexes in the absence of additional adjuvant. This strategy could also be used with QS-21, potentially decreasing the required amount of antigen and/or adjuvant in a vaccine formulation. We have already demonstrated that QS-21 can be directly coupled through an amide linkage through the glucuronic acid carboxyl to free amino groups on lysozyme, resulting in a 1:1 molar conjugate that induces higher antibody titers to lysozyme than free lysozyme and QS-21⁴. Additional studies are ongoing to analyze both antibody and CTL responses to a 1:1 molar covalent conjugate of OVA: QS-21.

In contrast to modification of glucuronic acid, the modification of the aldehyde at C4 on the QS-21 triterpene severely diminished adjuvant activity. All three derivatives modified at the aldehyde were inactive as adjuvants over a dose range 10-fold higher than the minimum dose of native QS-21 associated with some stimulatory effect ($2.5 \mu\text{g}$). Hence, this aldehyde may be critical to the adjuvant function. One possible mechanism involving the aldehyde might be the formation of a Schiff base with a free amino group on a cellular target to stabilize a cellular interaction. Stabilization of interaction of MHC Class II+ antigen-presenting cells and T_H cells via Schiff base interaction between free amino groups on antigen-presenting cells and aldehyde on the T cells has been noted²⁵. The inactivation of the QS-21 adjuvant function by blocking the aldehyde suggests that it may also be involved in a Schiff base interaction with a free amino group on the surface of an immune cell target. However, a direct Schiff base-stabilized interaction of QS-21 with a particular immune cell population has not yet been demonstrated. It was shown through *in vivo* and *in vitro* cell depletion and reconstitution studies that macrophages are critical for both induction of precursor CTL to QS-21/subunit antigen vaccines as well as being critical for antigen processing during CTL maturation²¹. Hence, macrophages may be an important site of action for QS-21. However, other immune cell populations such as T cells cannot be ruled

out. Additional studies are planned to further determine the site of action of QS-21. Upon determination of the immune cells that interact with QS-21, the possible interaction of the aldehyde with these cells will be explored.

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STRUCTURE OF DESACYLSAPONINS OBTAINED FROM THE BARK OF *QUILLAJA SAPONARIA*

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Key Word Index—*Quillaja saponaria*; Rosaceae; quillaja bark; quillajasaponin; triterpenoid saponin; desacyl-saponin; diazomethane degradation; quillaic acid 3,28-O-bisglycoside; quillaic acid.

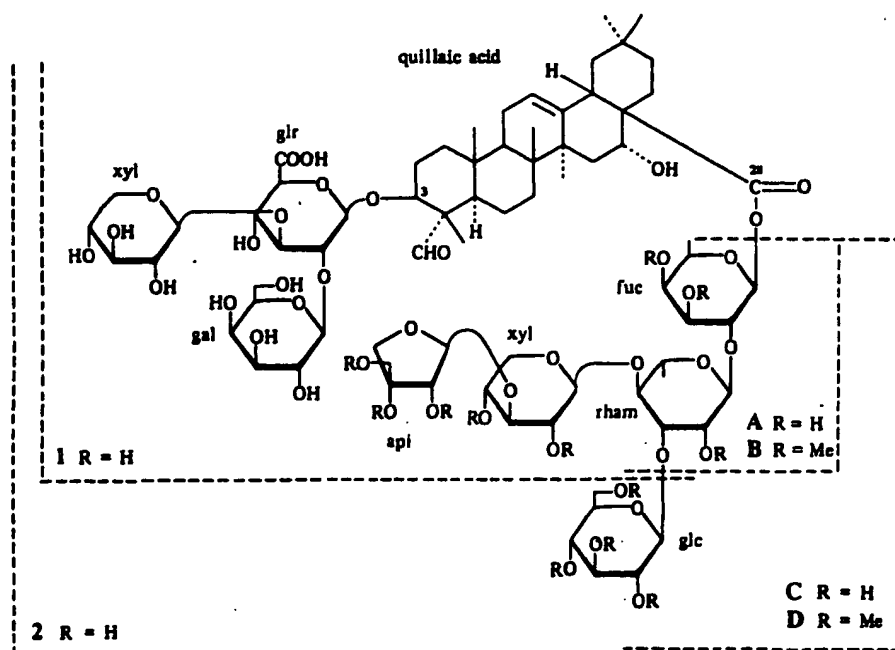
Abstracts—A triterpenoid saponin mixture (so-called quillajasaponin) obtained from the bark of *Quillaja saponaria* was treated with weak alkali and two major desacylsaponins were isolated. On the basis of chemical and spectral evidence, they were determined as 3-O- β -D-galactopyranosyl-(1 \rightarrow 2)-[β -D-xylopyranosyl-(1 \rightarrow 3)]- β -D-glucopyranosyl quillaic acid 28-O- β -D-apiofuranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-fucopyranoside and 28-O- β -D-apiofuranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 4)-[β -D-glucopyranosyl-(1 \rightarrow 3)]- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-fucopyranoside. Diazomethane degradation providing selectively the 28-O-glycoside from the 3,28-O-bisglycoside was a useful method for the structure elucidation.

INTRODUCTION

The bark of *Quillaja saponaria* Molina, named quillaja bark (cortex quillajae), is known as a saponin crude drug and has been used as a detergent, dentifrice and expectorant [1]. The existence of a saponin mixture (designated as quillajasaponin), which was recently reported to have a

strong adjuvant activity [2, 3] and a plasma cholesterol lowering effect [4], was recognized but as for the constituents of the crude saponin, little was known except for quillaic acid [5, 6] and its monoglucuronide [7], which were obtained upon acid hydrolysis of the saponin. A study on the constituents of the quillajasaponin has been conducted in an attempt to isolate the physiologically active triterpenoid compounds. We report in this paper the structures of two desacylsaponins (quillaic acid 3,28-O-bisglycosides), DS-1 (1) and DS-2 (2), obtained by mild alkaline hydrolysis of the quillajasaponin.

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RESULTS AND DISCUSSION

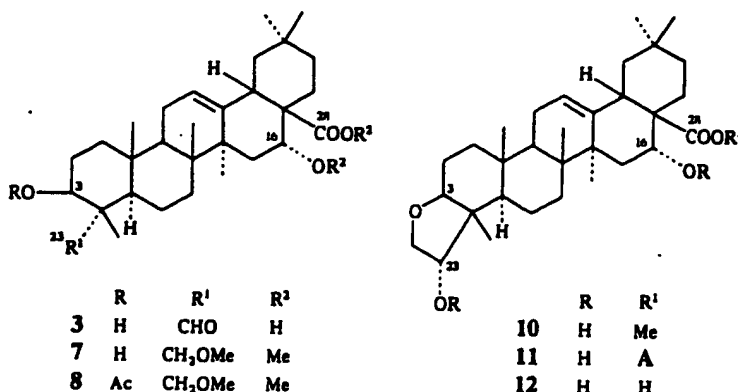
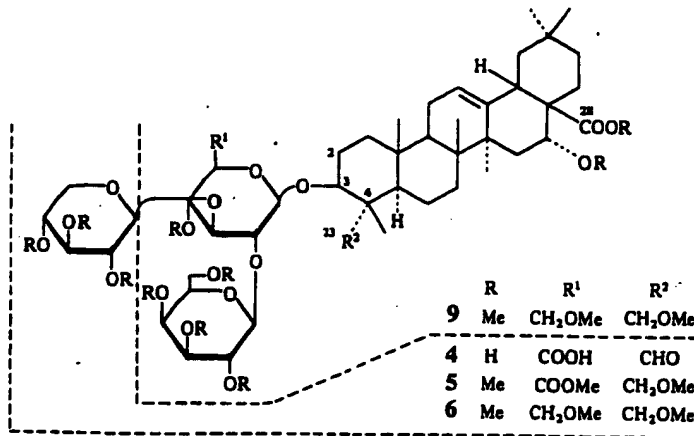
The methanol extract of the bark was fractionated by the ordinary procedure, as described in the Experimental, to give a saponin fraction (quillajasaponin). On treatment with 6% NaHCO_3 in 50% methanol, the fraction afforded a desacylsaponin mixture, which was separated by normal and reverse phase column chromatography to give two major compounds, DS-1 (1) and DS-2 (2). Compound 1, as well as 2, showed a single spot on normal and reverse phase TLC, respectively.

Compound 1 was hydrolysed with acid to yield glucuronic acid (Glr), galactose (Gal), xylose (Xyl), fucose (Fuc), rhamnose (Rham), apiose (Api) and quillaic acid (3). Compound 2 yielded the same products as those of 1 and in addition glucose (Glc). The ^{13}C NMR spectra of 1 and 2 showed seven and eight anomeric carbon signals respectively. The FAB mass spectra of 1 and 2 revealed the molecular ion peaks as a cationized cluster ion at m/z 1589 $[\text{M} + 2\text{K} - \text{H}]^+$ and 1751 $[\text{M} + 2\text{K} - \text{H}]^+$, respectively. These data indicate 1 to consist of 1 mol each of 3, Glr, Gal, Xyl, Fuc, Rham, Api and another one, Xyl or Api, and 2 to consist of the same components as those of 1 and 1 mol of Glc. Both compounds were suggested to have a 28-O-glycosidic linkage since in their ^{13}C NMR spectra

the signals due to C-28 of the aglycone part were observed at δ 176.0 (in 1) and 176.3 (in 2) [8].

When compounds 1 and 2 were treated with potassium hydroxide in 50% ethanol, they afforded the same prosapogenin (4), which was hydrolysed with acid to yield 3, Glr, Gal and Xyl. Compound 4 showed in the ^{13}C NMR spectrum three anomeric carbon signals and three carbon signals at δ 24.6, 54.9 and 84.2 due to C-2, C-3 and C-3' of quillaic acid having a sugar moiety at its position [9], and showed the molecular ion peak at m/z 995 $[\text{M} + \text{K}]^+$ in the FAB mass spectrum. These data indicate that 4 is the 3-O-glycoside of 3 and consists of 1 mol each of 3, Glr, Gal and Xyl. Since the aldehyde group and Glr unit in 4 were thought to complicate its structure elucidation, compound 4 was converted to the more stable compound 6 as follows.

Reduction of 4 with sodium borohydride followed by methylation of the product by the Hakomori method [10] afforded compound 5. Compound 5 was again reduced and methylated to yield compound 6, which was methylated to give an aglycone (7) and three methylated sugars. An acetate (8) of 7 showed in the ^1H NMR spectrum the signals of one acetoxyl and three methoxyl groups, together with triplet-like signals at δ 4.92 attributable to the proton at C-3 bearing the acetoxyl group.



	R	R ¹
10	H	Me
11	H	A
12	H	H
13	Me	B
14	Me	H
16	H	C
17	Me	D

Therefore 7 and 8 were regarded as the 16,23-di-*O*-methyl ether of 16 α -hydroxyhederagenin methyl ester and 16-*O*-acetate, respectively. The methylated sugars were identified as methyl pyranosides of 2,3,4-tri-*O*-methyl-glucose (S-1), 2,3,4,6-tetra-*O*-methyl-galactose (S-2) and 2,3,4,6-tetra-*O*-methyl-glucose (S-3). Therefore, compound 4 is a branched trisaccharide, xylopyranosyl-[galactopyranosyl]-glucuronopyranose, combined with the 3-hydroxyl group of 3, and the Xyl and Gal units are attached to the 2- and 3-, or 3- and 2-hydroxyl groups of 3, respectively.

When compound 6 was treated with dilute hydrochloric acid in methanol, a major product was obtained. Methylation of the product afforded compound 9, which on methanolysis compound 7, S-2 and methyl 3,4,6-tri-*O*-methyl-glucopyranose (S-4). This indicated compound 9 to be the permethylate of the galactosyl-(1 \rightarrow 2)-glucoside of 16 α -hydroxyhederagenin. Therefore, if Xyl, Glc and Glc are assumed to be the most commonly found series, the oligosaccharide moiety of 4 is D-xylopyranosyl-(1 \rightarrow 2)-[D-xylopyranosyl-(1 \rightarrow 3)]-D-glucuronopyranose. The ^1H NMR spectrum of 5 showed three anomeric proton signals of sugar units as doublets with large *J* values (7, 7 and 8 Hz) indicating their β -linkage [12].

Consequently, compound 4 is quillaic acid 3-*O*- β -D-xylopyranosyl-(1 \rightarrow 2)-[β -D-xylopyranosyl-(1 \rightarrow 3)]-D-glucuronopyranoside, and therefore compounds 1 and 2 are thought to be 28-*O*-glycosides of 4.

In the course of experiments concerning the structure elucidation of 4, we found and reported [13] that the aglycone linkage of 4 was cleaved to give the aglycone 10 (methyl 3 β ,16 α ,23 α -trihydroxy-3-*O*,23-dimethylenolean-12-en-28-oate) and the corresponding oligosaccharide residue by only treatment of 4 with diazomethane-ether in methanol. If this procedure (diazomethane degradation) is applied to compounds 1 and 2, the 28-*O*-glycoside of 12 must be obtained.

Treatment of compound 1 with diazomethane-ether in methanol afforded a less polar compound (11). Compound 11 revealed an ester carbonyl absorption (1735 cm^{-1}) in the IR spectrum, the signals of four anomeric carbons in the ^{13}C NMR spectrum and a molecular ion peak at *m/z* 1079 [*M* + Na] $^+$ in the FAB mass spectrum, and gave on acid hydrolysis Fuc, Rham, Xyl, Api and an aglycone (12), which was converted to 10 by methylation with diazomethane. These data indicated that 11 is the 28-*O*-tetraglycoside of 12 obtained by cleavage of the 3-*O*-glycosidic linkage in 1, and that the sugar moiety of 11 consisted of 1 mol each of Fuc, Rham, Xyl and Api.

Methanolysis of the permethylate (13) of 11 gave 14 (the 16,23-di-*O*-methyl ether of 12), methyl 2,3,5-tri-*O*-methyl-apiofuranoside (S-5) and methyl pyranosides of 2,4-di-*O*-methyl-xylose (S-6), 2,3-di-*O*-methyl-rhamnose (S-7) and 3,4-di-*O*-methyl-fucose (S-8). This indicated that the sugar moiety of 11 is linear in structure and that apiofuranose is located at the terminal. Compound 11 was treated with 2% hydrochloric acid in methanol to yield 15 together with 12 and methyl apiofuranoside. Compound 15 showed a molecular ion peak at *m/z* 479 [*M* + Na] $^+$ in the FAB mass spectrum and revealed one methoxyl and three anomeric carbon signals in the ^{13}C NMR spectrum, and afforded Xyl, Rham and Fuc on acid hydrolysis. These data indicated that 15 was a methyl glycoside of a trisaccharide consisting of Xyl, Rham and Fuc. The

detailed assignment of the ^{13}C NMR spectrum of 15 was made by taking the glycosylation shift [14, 15] into account and by comparison with the spectra of methyl fucopyranoside and gleditsia saponin [16] as shown in Table 1. The result of methanolysis of 13 indicated that if Xyl, Fuc and Rham are assumed to be the most commonly found D, D and L-series, compound 15 is the methyl glycoside of β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -D-fucopyranose. The above facts suggested that the sugar moiety of 11 is D-apiofuranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)-D-fucopyranose since apiose in glycosides is also usually found as the D-series.

The configuration of the D-fucopyranose unit was regarded to be β by the *J* values of its anomeric proton signal (doublet, *J* = 8 Hz) [12] in the ^1H NMR spectrum of 13. The D-apiofuranose unit was considered to have the β -configuration by comparison of its anomeric carbon signal (δ 111.1) in the ^{13}C NMR spectrum of 11 with those of the methyl β - and α -D-apiofuranosides (β -anomer: δ 111.3; α -anomer: δ 104.4). Therefore, it follows that compound 11 is the 28-*O*- β -D-apiofuranosyl-(1 \rightarrow 3)- β -D-

Table 1. ^{13}C NMR spectral data ($\text{C}_3\text{D}_3\text{N}$) of compounds 15, 18 and reference compounds

C	15	18	Reference compounds*
1'	100.5	100.4	[F]
2'	78.4	77.9†	70.0
3'	69.9	69.9	71.6
4'	73.3	73.2	73.2
5'	66.6	66.6	66.9
6'	17.0	17.0	17.1
OMe	54.9	54.9	55.1
1"	104.2	104.0	[R]
2"	71.6	71.1	71.9
3"	72.7	77.7†	72.6
4"	84.5	82.7	83.9
5"	68.1	68.1	68.4
6"	18.4	18.7	18.7
1"	107.0	105.1‡	[X]
2"	76.0	75.4§	106.9
3"	78.5	78.7	76.0
4"	70.9	71.1	78.6
5"	67.4	67.0	71.0
6"			67.4
1"		104.7‡	[G]
2"		75.2§	105.5
3"		78.7	74.9
4"		71.1	78.3
5"		78.7	71.6
6"		62.2	78.3
			62.7

*[F]: Methyl α -D-fucopyranoside; [R] and [X]: rhamnose and xylose parts in β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl moiety in gleditsia saponin [16]; [G]: methyl β -D-glucopyranoside.

†,‡,§ Assignments may be reversed in each vertical column.

xylopyranosyl-(1 → 4)- α -L-rhamnopyranosyl-(1 → 2)- β -D-fucopyranoside of 12.

Consequently, due to the structures of compounds 4 and 11, compound 1 is characterized as 3-O- β -D-galactopyranosyl-(1 → 2)-[β -D-xylopyranosyl-(1 → 3)]- β -D-glucuronopyranosyl quillaic acid 28-O- β -D-apiofuranosyl-(1 → 3)- β -D-xylopyranosyl-(1 → 4)- α -L-rhamnopyranosyl-(1 → 2)- β -D-fucopyranoside.

The structure of the 28-O-oligosaccharide moiety in compound 2 was also determined by using the diazomethane degradation method as follows. On treatment with diazomethane, compound 2 afforded a less polar compound (16) which was presumed to be the 28-O-pentaglycoside of 12. On acid hydrolysis 16 gave 12, Fuc, Rham, Xyl, Api and Glc, and showed the molecular ion peak at m/z 1241 $[M + Na]^+$ in the FAB mass spectrum, five anomeric carbon signals in the ^{13}C NMR spectrum and an ester carbonyl absorption (1735 cm^{-1}) in the IR spectrum. These data indicated 16 is the 28-O-glycoside of 12 consisting of 1 mol each of 12, Fuc, Rham, Xyl, Api and Glc. Methanolysis of the permethylate (17) of 16 afforded 14 and five methylated sugars, S-5, S-6, S-8 and the methyl pyranosides of 2-O-methyl-rhamnose (S-9) and 2,3,4,6-tetra-O-methyl-glucose (S-10). These facts and the co-existence of 1 and 2 in the same plant material suggested that the sugar moiety of 16 is a pentasaccharide in which a glucopyranose is located on the 3-hydroxyl group of the rhamnopyranose unit in the sugar moiety of 11.

When compound 16 was treated with 2% hydrochloric acid in methanol in the same manner as 11, compound 18 was obtained together with 12 and methyl apiofuranoside. Since 18 gave on acid hydrolysis Xyl, Rham, Fuc and Glc and showed the signals of one methoxyl and four anomeric carbons in the ^{13}C NMR spectrum and the molecular ion peak at m/z 619 $[M + H]^+$ in the FAB mass spectrum, compound 18 was regarded as the methyl glycoside of tetraose derived from the sugar moiety of 16. The ^{13}C NMR signals of 18 were assigned as shown in Table 1 by taking the glycosylation shift into account and by comparison with the spectra of 15 and methyl glucopyranoside. The permethylate (19) of 18 was methanolysed to give S-8, S-9, S-10 and S-1. Therefore, if Xyl, Glc, Fuc and Rham are assumed to be the commonly found D, D, D and L-series, compound 18 must be methyl β -D-xylopyranosyl-(1 → 4)-[β -D-glucopyranosyl-(1 → 3)]- α -L-rhamnopyranosyl-(1 → 2)- α -D-fucopyranoside. The site

of linkage of the Glc and Xyl units to the 3- and 4-hydroxyl groups of the rhamnose unit in 18 was confirmed as follows. Compound 19 was hydrolysed with 5% hydrochloric acid in methanol and the major product was methylated to give a compound (20) which showed no hydroxyl absorption in the IR spectrum and three anomeric proton signals in the 1H NMR spectrum. Methanolysis of 20 afforded three methylated sugars, S-5, S-10 and methyl 2,4-di-O-methyl-rhamnopyranoside (S-11), which indicated that 20 is the permethylate of glucosyl-(1 → 3)-rhamnosyl-(1 → 2)-fucose.

Accordingly, compound 16 is the 28-O-apiofuranosyl-(1 → 3)- β -D-xylopyranosyl-(1 → 4)-[β -D-glucopyranosyl-(1 → 3)]- α -L-rhamnopyranosyl-(1 → 2)-D-fucopyranoside of 12. The configurations of both D-fucopyranose and D-apiofuranose were suggested to be β by the anomeric proton signal of fucopyranose (doublet, $\tau = 8\text{ Hz}$) [12] in the 1H NMR spectrum of 17 and the anomeric carbon signals of apiofuranose (δ 111.2) in the ^{13}C NMR spectrum of 16.

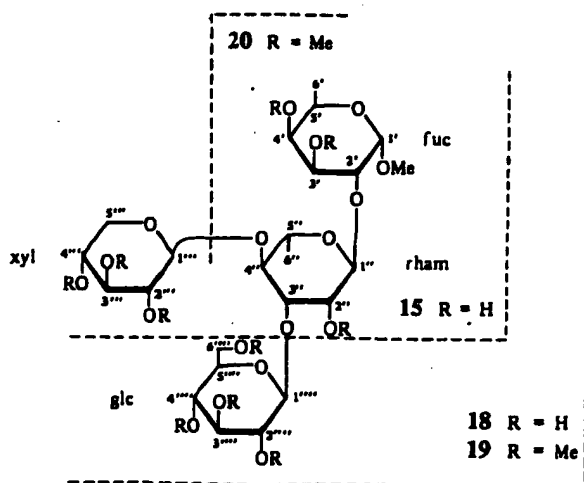
Based on the structure of 16, compound 2 was regarded to be 3-O- β -D-galactopyranosyl-(1 → 2)-[β -D-xylopyranosyl-(1 → 3)]- β -D-glucuronopyranosyl quillaic acid 28-O- β -D-apiofuranosyl-(1 → 3)- β -D-xylopyranosyl-(1 → 4)-[β -D-glucopyranosyl-(1 → 3)]- α -L-rhamnopyranosyl-(1 → 2)- β -D-fucopyranoside.

To our knowledge, two quillaic acid 3,28-O-bisglycosides have been reported [9, 17], but the two desacylsaponins, 1 and 2, reported here are different in their sugar moieties from those described so far. Diazomethane degradation was a useful method for structure elucidation of the triterpene 3,28-O-bisglycoside as described in this paper.

EXPERIMENTAL

All mps are uncorr. Optical rotations were recorded at 18–28° using a 1 dm cell. 1H NMR spectra were taken at 100 MHz in $CDCl_3$ soln unless otherwise specified, using TMS as internal standard. ^{13}C NMR spectra were recorded at 25 MHz in C_2D_2N (TMS as internal standard) unless otherwise noted, employing the FT mode. The EI- and FABMS were measured on a double focusing mass spectrometer. The former were taken with an accelerating potential of 3–6.5 kV and an ionizing potential of 30–75 eV, and the latter at 1.5–3 kV for the ion source and 6 kV for an Ar beam source; the spectra were obtained from glycerol solns unless otherwise specified. FDMS were taken at 2–3 kV for the field anode and at –5 kV for the slotted cathode plate, at an ion source pressure of $ca\ 10^{-7}$ Torr and an emitter heating current of 18–24 mA. Conditions of GLC (FID mode): (a) glass column (1.2 m \times 3 mm) packed with 10% 1,4-butanediol succinate on Shimalite W (60–80 mesh), column temp. 155°; (b) glass column (1.2 m \times 3 mm) packed with 1% neopentyl glycol succinate polyester on Chromosorb W(AW)-DMCS (60–80 mesh), column temp. 130°. Solvent systems of TLC [silica gel, C-8 (reversed phase) and Avicel]: (a) $CHCl_3$ -MeOH-HOAc-H₂O (15:9:1:2); (b) EtOAc-MeOH-H₂O (8:1:0.1); (c) $CHCl_3$ -MeOH (10:1); (d) C_6H_6 -Me₂CO (2:1); (e) C_6H_6 -Me₂CO (4:1); (f) *n*-hexane-EtOAc (2:1); (g) 60% MeOH; (h) upper layer of *n*-BuOH-pyridine-H₂O (6:2:3) + pyridine (1); (i) upper layer of *n*-BuOH-HOAc-H₂O (4:1:5).

Isolation of quillajasaponin. Commercial quillaja bark (2.5 kg) (a voucher specimen of the crude drug is on file at the Faculty of Pharmaceutical Sciences, Kyushu University) was extracted with MeOH and the MeOH was evaporated *in vacuo* to leave the MeOH extract (420 g). The latter was partitioned between EtOAc



H₂O, and the H₂O layer was passed through an Amberlite MD-2 column and eluted with H₂O and MeOH. Crude saponins (85 g) obtained by evaporation of the MeOH eluate were treated with Sephadex LH-20 CC (eluate MeOH) to give two fractions, fraction 1 (39 g) (quillajasaponin) and 2 (42 g). Fraction 1 showing 7 spots (giving a dark-green colour after spraying with 5% H₂SO₄-MeOH followed by heating) on TLC [silica gel (a)] was refluxed with 3.5% HCl in 50% EtOH for 3 hr and the reaction mixture revealed a spot of quillaic acid (*R_f* 0.40) on TLC [silica gel (c)] (identified with an authentic sample). Fraction 2 showed two major spots (yellow colour by 5% H₂SO₄-MeOH and heating) on TLC [silica gel (a)] and further investigation was not carried out.

Alkaline hydrolysis of quillajasaponin. Quillajasaponin (18 g) was boiled with 6% NaHCO₃ in 50% MeOH (300 ml) for 1 hr, and the reaction mixture was neutralized with Dowex 50W-X8 and filtered. The filtrate showing spots of less polar and polar compounds on TLC [silica gel (a)] was evaporated *in vacuo* and the residue was chromatographed on Sephadex LH-20 (MeOH) to give two fractions, fractions 1 (5.0 g) (polar compounds, acylsaponins) and 2 (2.2 g) (less polar compounds, eliminated acyl groups). Fraction 1 showing two major spots (*R_f* 0.11, 0.07) on TLC [silica gel (a)] was chromatographed on silica gel (eluant CHCl₃-MeOH-HOAc-H₂O, 15:9:1:2) to give two fractions, fractions 1' (*R_f* 0.11) and 2' (*R_f* 0.07). Each fraction showed a major and a few minor spots on TLC [C-8(g)] and each was chromatographed on C-8 (eluant 50% MeOH) to give a major component, DS-1 (1, 1.4 g) and DS-2 (2, 2.0 g) [*R_f* 0.11 (1) and 0.07 (2), silica gel (a); *R_f* 0.29 (1) and 0.35 (2), C-8 (g)].

DS-1 (1). White powder (from *n*-BuOH-H₂O-MeOH), mp 255-258° (decomp.), [α]_D -19.6° (H₂O; *c* 0.67). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400 (OH), 1730 (carbonyl), 1610 (COO⁻). FABMS (using KI as additive) *m/z*: 1589 [M(C₆₅H₁₀₈O₃₆) + 2K - H]⁺ [(C₆₅H₁₀₇O₃₄ · COOK) + K]⁺. ¹³C NMR: δ 94.8, 101.0, 103.1, 103.9, 104.5, 106.9, 111.0 (each *d*, anomeric C × 7), 176.0 (*s*, C-28), 209.8 (*d*, C-23). Compound 1 (40 mg) was refluxed with 2 N H₂SO₄ in 50% EtOH for 8 hr and then diluted with H₂O, and the ppt was collected by filtration. The ppt. (crude aglycone) (10 mg) was chromatographed on silica gel (CHCl₃-MeOH, 30:1 (5:1) to provide 3 as colourless needles (from MeOH-H₂O), mp 256-260°, [α]_D +66.7° (MeOH; *c* 1.02). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3450 (OH), 1730, 1715 (carbonyl). EIMS *m/z*: 486 [M(C₃₀H₄₆O₅)]⁺, 264, 246. Compound 3 was identified with an authentic sample of quillaic acid, by mmp, TLC, IR and ¹³C NMR. The H₂O layer was neutralized with Ba(OH)₂ soln, filtered and the filtrate was concentrated. The residue was examined by TLC [Avicel (h) and (i)], and Glc, Gal, Xyl, Fuc, Rham and Api were detected.

DS-2 (2). White powder (from *n*-BuOH-H₂O-MeOH), mp 258-261° (decomp.), [α]_D -24.7° (H₂O; *c* 1.00). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400 (OH), 1730 (carbonyl), 1610 (COO⁻). FABMS (using KI as additive) *m/z*: 1751 [M(C₇₃H₁₁₈O₄₁) + 2K - H]⁺ [(C₇₃H₁₁₇O₃₉ · COOK) + K]⁺. ¹³C NMR: δ 95.0, 101.9, 103.1, 103.6, 104.3, 104.5, 104.9, 110.8 (each *d*, anomeric C × 8), 176.3 (*s*, C-28), 210.2 (*d*, C-23). On hydrolysis with acid under the same conditions as those for 1, compound 2 gave 3 and a sugar mixture. The sugar mixture was found to consist of Glc, Gal, Xyl, Fuc, Rham, Api and Glc [TLC, Avicel (h) and (i)].

Alkaline hydrolysis of 1 and 2 providing 4. Compound 1 (200 mg) was refluxed with 2% KOH in 50% EtOH (10 ml) for 1 hr and the reaction mixture was neutralized with dilute HCl and concentrated *in vacuo*. The residue showing a spot (*R_f* 0.40) on TLC [silica gel (a)] was passed through a Sephadex G-15 column (H₂O) and a silica gel column (CHCl₃-MeOH-HOAc-H₂O, 15:9:1:2) to give a white powder (4) (from *n*-BuOH-H₂O-MeOH) (80 mg), mp > 300° (decomp.), [α]_D -18.6° (H₂O; *c* 0.73). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400 (OH), 1710 (carbonyl),

1610 (COO⁻). FABMS *m/z*: 995 [M(C₄₇H₇₂O₂₀) + K]⁺. ¹³C NMR (C₅D₅N + D₂O): δ 24.6 (*t*, C-2), 54.9 (*s*, C-4), 84.2 (*d*, C-3), 102.7, 102.9, 103.6 (each *d*, anomeric C × 3), 180.1 (*s*, C-28), 210.1 (*d*, C-23). Compound 4 was hydrolysed with acid in the same manner as for 1 to provide 3 and sugars. The sugars were identified as Glc, Gal and Xyl [TLC, Avicel (h) and (i)]. Compound 2 (500 mg) was hydrolysed with alkali and worked up in the same manner as that for 1 to provide a white powder (200 mg) which was identical with 4 (mmp, TLC, IR and ¹³C NMR).

Preparation of 6 from 4. NaBH₄ (1.6 g) was added to a soln of 4 (250 mg) in 50% MeOH (25 ml) under ice-cooling and the reaction mixture was stirred for 2 hr at room temp. Me₂CO (2 ml) was added to the mixture and concentrated *in vacuo*, and the residue was passed through a Diaion HP20AG column (eluant H₂O → MeOH). The MeOH eluate showing a spot (*R_f* 0.36) on TLC [silica gel (a)] was evaporated *in vacuo* to give a white powder (230 mg), mp 275-277° (decomp.), [α]_D +3.8° (H₂O; *c* 0.9). ¹³C NMR: δ 64.2 (*t*, C-23), 175.0 (*s*, COOH of Glc), 182.4 (*s*, C-28), no CHO. The product (200 mg) was treated with NaH (500 mg) and MeI (5 ml) in DMSO (20 ml) (Hakomori method) and the reaction mixture was diluted with H₂O, extracted with Et₂O and the Et₂O layer was washed, dried and evaporated. The residue was chromatographed on silica gel (C₆H₆-Me₂CO, 7:1) to give a major product as a white powder (5) (150 mg). IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm⁻¹: 1760 (Glc-COOMe) [11], 1720 (28-COOMe) [18], no OH. ¹H NMR: δ 4.35 (1H, *d*, *J* = 7 Hz, anomeric H), 4.87 (1H, *d*, *J* = 7 Hz, anomeric H), 4.96 (1H, *d*, *J* = 8 Hz, anomeric H). FDMS *m/z*: 1127 [M(C₅₉H₉₈O₂₀) + H]⁺. Compound 5 (100 mg) in MeOH (10 ml) was reduced with NaBH₄ (1.2 g) as for 4. Me₂CO (2 ml) and H₂O (2 ml) were added to the mixture and evaporated *in vacuo*, and the residue was diluted with excess H₂O and extracted with CHCl₃. The CHCl₃ layer was washed, dried and concentrated, and the residue showing a spot (*R_f* 0.53) (*R_f* of 5, 0.77) on TLC [silica gel (d)] was methylated by the Hakomori method. The crude methylated product was chromatographed on silica gel (C₆H₆-Me₂CO, 7:1) to give 6 as a white powder (23 mg). IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm⁻¹: 1720 (28-COOMe), no OH. ¹H NMR: δ 3.30 (6H, *s*, OMe × 2), 3.35, 3.37, 3.46 (each *s*, 3H, OMe × 3), 3.50 (9H, *s*, OMe × 3), 3.53, 3.55 (each *s*, 3H, OMe × 2), 3.59 (6H, *s*, OMe × 2), 4.24 (1H, *d*, *J* = 7 Hz, anomeric H), 4.92 (1H, *d*, *J* = 7 Hz, anomeric H), 5.02 (1H, *d*, *J* = 8 Hz, anomeric H).

Methanolysis of 6. Compound 6 (35 mg) was boiled with 10% HCl in MeOH (4 ml) for 2 hr, the mixture was neutralized with Ag₂CO₃ and filtered. The filtrate was evaporated and the residue (methanolysate) was examined by TLC [silica gel (f) and (d)] and GLC [conditions (a) and (b)]; one aglycone (7) and three methylated sugars were detected. The sugars were identified as S-1, S-2 and S-3 by direct comparison with authentic samples. The methanolysate was chromatographed on silica gel (C₆H₆-Me₂CO, 80:1 → Me₂CO) to give 7 (10 mg). Compound 7 was acetylated with Ac₂O-pyridine as usual to give an acetate (8) (5 mg) as colourless needles (from MeOH-Me₂CO), mp 207-210°. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm⁻¹: 1730 (ester), no OH. EIMS *m/z*: 572 [M(C₃₅H₅₆O₆)]⁺, 292. ¹H NMR: δ 2.04 (3H, *s*, OAc), 2.93 and 3.10 (1H each, *d*, *J* = 10 Hz, H₁-23), 3.25, 3.29, 3.59 (each *s*, 3H, OMe × 3), 3.91 (1H, *br s*, H-16), 4.92 (1H, *t*-like, *J* = 8 Hz, H-3).

Preparation and methanolysis of 9. Compound 6 was heated with 2% HCl-MeOH for 8 hr at 60° and worked up as before. The mixture showing two major spots (*R_f* 0.28(6), 0.14) on TLC (silica gel; C₆H₆-Me₂CO, 5:1) was chromatographed on silica gel (*n*-hexane-Me₂CO, 5:1) to give a compound (*R_f* 0.14) which was methylated by the Hakomori method and worked up as before to yield 9 as a white powder. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm⁻¹: 1720 (ester), no OH. ¹H NMR: δ 3.28 (6H, *s*, OMe × 2), 3.32, 3.35, 3.39 (each *s*, 3H, OMe × 3), 3.51 (9H, *s*, OMe × 3), 3.60, 3.65 (each *s*, 3H, OMe × 2),

4.24 (1H, *d*, *J* = 8 Hz, anomeric H), 4.62 (1H, *d*, *J* = 7 Hz, anomeric H). Compound 9 was subjected to methanolysis as for 6, and 7: S-2 and S-4 were obtained [TLC silica gel (f), GLC (a)].

Diazomethane degradation of 1 providing 11. A soln of CH₂N₂ in Et₂O (150 ml) was poured into a soln of 1 (1.1 g) in MeOH (300 ml) under cooling with ice. The mixture was left to stand for 1 hr at room temp., excess CH₂N₂ was decomposed with HOAc and the solvents were removed by distillation. The crude reaction mixture revealing two major spots (*R_f* 0.51 and 0.27) in TLC [silica gel (a)] was chromatographed on silica gel (CHCl₃-MeOH-H₂O, 8:3:0.3) to give a substance (white powder, *R_f* 0.27) (a mixture of the related compounds of the methyl ester of 1 and trisaccharides derived from the 3-*O*-sugar residue in 1 [13]) and 11 (white powder, 164 mg, *R_f* 0.51), mp 198–201° (decomp.), $[\alpha]_D^{20}$ –30.4° (MeOH; *c* 1.65). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{–1}: 3400 (OH), 1735 (ester). FABMS *m/z*: 1079 [M(C₃₃H₅₄O₃₁) + Na]⁺. ¹³CNMR: δ 94.8, 101.2, 106.9, 111.1 (each *d*, anomeric C \times 4), 176.1 (*s*, C-28). Compound 11 was boiled with 2N H₂SO₄ for 2 hr and the reaction mixture was extracted with CHCl₃. The CHCl₃ layer showing a spot (*R_f* 0.21) on TLC [silica gel (e)] was washed, dried and evaporated to give 12, which was methylated with CH₂N₂-Et₂O in MeOH affording a compound corresponding with an authentic sample of 10 [TLC (e), IR, ¹H NMR and ¹³C NMR]. The aq. layer was treated as for 1 and Fuc, Rham, Xyl and Api were detected [TLC, Avicel (h) and (i)].

Synthesis and methanolysis of the permethylate (13) of 11. Compound 11 (40 mg) was methylated by the Hakomori method as before, and the reaction mixture was diluted with H₂O, extracted with CHCl₃ and the CHCl₃ layer was washed, dried and evaporated. The residue was passed through silica gel (*n*-hexane-Me₂CO, 2:1) and a Sephadex LH-20 (MeOH) column to give 13 as a white powder (16 mg). IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{–1}: 1750 (ester), no OH. FDMS *m/z*: 1210 [M(C₆₄H₁₀₆O₂₁)]⁺. ¹H NMR: δ 4.63 (1H, *d*, *J* = 8 Hz, anomeric H of Xyl), 5.24 (1H, *d*, *J* = 1 Hz, anomeric H of Rham), 5.37 (1H, *d*, *J* = 8 Hz, anomeric H of ester glycosidic fucose [19]), 5.46 (1H, *d*, *J* = 2 Hz, anomeric H of Api [20]) (anomeric H signals were assigned by comparison with those observed in the ¹H NMR spectrum of 17, and with the reported δ and *J* values [19, 20]). Compound 13 was methanolysed and worked up in the same manner as that for 6. The methanolysate was examined by TLC [silica gel (d)] and GLC [condition (a)], and S-5, S-6, S-7 and S-8 were detected. Silica gel CC of the methanolysate (*n*-hexane-Me₂CO, 6:1) afforded an aglycone (14). EIMS *m/z*: 528 [M(C₃₃H₅₂O₃)]⁺, 278.

Partial methanolysis of 11 yielding 15. Compound 11 (300 mg) in 2% HCl-MeOH (20 ml) was left to stand for 2.5 hr at 40°, and the reaction mixture was treated with Ag₂CO₃, filtered and evaporated. The residue giving three spots [*R_f* 0.84(12), 0.61 (methyl β -D-apiofuranoside) and 0.31] on TLC [silica gel (a)] was chromatographed on silica gel (CHCl₃-MeOH-HOAc-H₂O, 15:8:1:1) and Sephadex LH-20 (MeOH) to yield 15 (*R_f* 0.31) (31 mg) as a hygroscopic powder, $[\alpha]_D^{20}$ +11.8° (MeOH; *c* 1.00). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{–1}: 3400 (OH). FAB MS *m/z*: 479 [M(C₁₈H₃₂O₁₃) + Na]⁺. ¹³CNMR: see Table 1. Compound 15 was boiled with 2N H₂SO₄ for 2 hr and treated as usual. The hydrolysate was examined by TLC [Avicel (h) and (i)], and Xyl, Rham and Fuc were detected.

Diazomethane degradation of 2 affording 16. Compound 2 (2.0 g) in MeOH (150 ml) was treated with CH₂N₂-Et₂O (130 ml) and worked up as for 1. The crude reaction mixture showing three spots (*R_f* 0.42, 0.27 and 0.21) on TLC [silica gel (a)] was chromatographed on silica gel (CHCl₃-MeOH-H₂O, 6:4:0.3) to give substances of *R_f* 0.27 (3-*O*-trisaccharide residues in 2), *R_f* 0.21 (related compounds of the methyl ester of 2) and *R_f* 0.42 (16) (a white powder, 508 mg), mp 211–214° (decomp.), $[\alpha]_D^{20}$ –40.4° (MeOH; *c* 3.23). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{–1}: 3420 (OH), 1735 (ester).

FAB MS *m/z*: 1241 [M(C₅₉H₉₄O₂₆) + Na]⁺. ¹³CNMR: δ 95.1, 102.0, 104.8, 105.2, 111.2 (each *d*, anomeric C \times 5), 175.9 (*s*, C-28). On hydrolysis with acid under the same conditions as for 11, 16 gave 12 and a sugar mixture. The sugar mixture was found to consist of Fuc, Rham, Xyl, Api and Glc [TLC, Avicel (h) and (i)].

Preparation and methanolysis of 17. Compound 16 (300 mg) was methylated by the Hakomori method and worked up in the same manner as for 11 to provide a permethylate (17) (88 mg) as a white powder. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{–1}: 1755 (ester), no OH. FDMS *m/z*: 1414 [M(C₇₃H₁₂₂O₂₆)]⁺. ¹H NMR: δ 4.68 (1H, *d*, *J* = 7 Hz, anomeric H of xylose), 4.82 (1H, *d*, *J* = 7 Hz, anomeric H of Glc), 5.05 (1H, *d*, *J* = 2 Hz, anomeric H of Rham), 5.39 (1H, *d*, *J* = 8 Hz, anomeric H of Fuc), 5.41 (1H, *d*, *J* = 2 Hz, anomeric H of Api) (anomeric H signals were assigned by comparison with the ¹H NMR spectrum of 13). Compound 17 was methanolysed and the methanolysate was examined as for 13. Compound 14, S-5, S-6, S-8, S-9 and S-10 were detected [TLC silica gel (d), GLC (a)].

Partial methanolysis of 16 yielding 18. Compound 16 (450 mg) was treated with 2% HCl-MeOH for 1.5 hr and worked up as for 11. The residue showing the spots of 12, methyl apiofuranoside and 18 (*R_f* 0.22) on TLC [silica gel (a)] was chromatographed on silica gel (EtOAc-MeOH-H₂O, 6:4:0.4) and Sephadex LH-20 (MeOH) to give 18 (145 mg) as a white powder, mp 178–182° ($[\alpha]_D^{20}$ –3.2° (MeOH; *c* 3.73). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{–1}: 3380 (OH). FABMS *m/z*: 619 [M(C₂₄H₄₂O₁₁) + H]⁺. ¹³CNMR: see Table 1. Compound 18 was hydrolysed with acid and worked up as for 15. Xyl, Glc, Rham and Fuc were obtained [TLC Avicel (h) and (i)].

Synthesis of 19 and its methanolysis. In the same manner as that for 11, compound 19 (63 mg) was prepared from 18 (120 mg) by the Hakomori method. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{–1}: no OH. EIMS *m/z*: 758 [M(C₃₄H₆₂O₁₈)]⁺. Compound 19 was methanolysed as for 13 to give S-8, S-9, S-10 and S-11 [TLC silica gel (d), GLC (a)].

Preparation of 20 and its methanolysis. Compound 19 (50 mg) was methanolysed with 5% HCl-MeOH (3 ml) for 3 hr at 50° and worked up as before, and the residue (partial methanolysate) was chromatographed on silica gel (*n*-hexane-Me₂CO, 3:1) to give a major product (*R_f* 0.09) [TLC silica gel (*n*-hexane-Me₂CO, 3:1)] (*R_f* of 19, 0.18). The major product was methylated by the Hakomori method and treated as usual to yield 20 (8 mg) as a colourless syrup. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{–1}: no OH. ¹H NMR: δ 3.33, 3.34, 3.47, 3.53, 3.57, 3.60, 3.62 (each *s*, 3H, OMe \times 7), 3.52 (6H, *s*, OMe \times 2), 4.45 (1H, *d*, *J* = 7 Hz, anomeric H), 4.77 (1H, *d*, *J* = 4 Hz, anomeric H), 4.95 (1H, *d*, *J* = 2 Hz, anomeric H). Methanolysis of 20 as before afforded S-8, S-10 and S-11 [TLC silica gel (d), GLC (a)].

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QS-21 structure/function studies: effect of acylation on adjuvant activity

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Abstract

QS-21 is a natural saponin adjuvant derived from the tree *Quillaja saponaria* Molina. Previous studies over a limited dose range suggested the acylation is critical to adjuvant activity. In this study, we prepared DS-1 (deacylated QS-21) and RDS-1 (reacylated DS-1 with dodecylamine at a different site than QS-21) to determine the effect on a dose-response curve over a wider range in mice. DS-1 and RDS-1 induced IgG1 responses at higher doses compared to that induced by QS-21. DS-1 was inactive for inducing IgG2a or CTL responses at any doses. RDS-1 showed moderate IgG2a response at 240 μ g, but did not show CTL response at any dose evaluated. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Adjuvant; QS-21; Structure/function; DS-1; GPI-0100

1. Introduction

QS-21 is a highly purified immunological adjuvant derived from the bark of the South American tree *Quillaja saponaria* Molina [1–4]. It is a water soluble triterpene glycoside with amphiphilic character that can be mixed with a soluble antigen resulting in a fully soluble vaccine formulation or that can be combined with emulsion or mineral salt adjuvants. This molecule promotes both humoral and cell-mediated immunity when added to parenteral or mucosal vaccine formulations [5–11]. QS-21 is currently under clinical evaluation for various vaccines [12] and has been tested in more than 3000 patients in 60 clinical trials. In recent clinical studies, QS-21 was shown to be more effective than aluminum hydroxide in stimulation of an antibody response to a malaria peptide vaccine [13] and to low dose HIV-1 gp120 [14]. QS-21 has also been used in clinical vaccines to induce cellular immune responses. A tyrosinase peptide vaccine containing QS-21 was shown to induce CD8⁺ T-cells in a subset of patients in a metastatic melanoma clinical study [15].

In contrast to the majority of saponins from other species, *Quillaja* saponins are acylated. The three most predominant saponins (QS-17, QS-18 and QS-21) are acylated at the 4-hydroxyl position of fucose with two linked 3,5-dihydroxy-6-methyloctanoic acids containing a glycosylation site at the 5-OH position of one of the acyl chains. This acylation may be critical for adjuvant activity. Deacy-

lated QS-18 and QS-21, each evaluated in mice at a 10 μ g dose, were shown to induce a lower total IgG response to bovine serum albumin than that induced by the native acylated forms [2]. The deacylsaponin of QS-21 (termed DS-1) was evaluated as an adjuvant for antibody response (over the dose range of up to 40 μ g) and CTL response (single dose of 10 μ g) against ovalbumin in mice [16]. In contrast to QS-21, DS-1 did not stimulate a strong level of antibody (measured as total anti-OVA IgG) or OVA-specific CTL responses. The corresponding fatty acid fragment also appeared to be inactive [16]. Recently, Marciani et al. [17] reported on a preparation of semisynthetic triterpenoid saponins known as GPI-0100. GPI-0100 was prepared by deacylating a crude mixture of *Q. saponaria* saponins and then coupling dodecylamine with the carboxyl group of the glucuronic acid residue of the deacylated saponins through an amide bond. GPI-0100 was used as a reaction mixture without further purification. It was reported that GPI-0100 can stimulate a Th1 antibody isotype profile (IgG2a) as well as CTL production against exogenous antigens. A dose of >200 μ g of GPI-0100 was equivalent to a dose of 10 μ g of the corresponding native saponins for CTL response to OVA. The adjuvant activity of the deacylsaponin intermediates was not reported.

In this study, we compared the adjuvant activity of DS-1 (deacylated QS-21) and deacylated crude saponins (crude deacylsaponins) to QS-21 over a broad dose range. RDS-1 (HPLC purified GPI-0100 analogue of QS-21) and GPI-0100 were prepared and compared in an effort to understand the importance of acylation to the adjuvant activity of *Quillaja* saponins.

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2. Materials and methods

2.1. Synthesis of saponin analogues

Quillaja saponins were extracted from coarsely chopped *Q. saponaria* bark by previously described method [1]. *N,N*-dicyclohexyl-carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dodecylamine and pyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Ethyl acetate, acetonitrile and water were obtained from VWR Scientific (Boston, MA, USA). Triethylamine and trifluoroacetic acid (TFA) were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA) and Pierce (Rockford, IL, USA) respectively. All solvents were HPLC grade. Electrospray ionization (ESI) mass spectra were measured at Mass Consortium (San Diego, CA, USA). NMR spectra were recorded on a Varian-400 NMR Instrument at Jing Hong Custom NMR services (Cambridge, MA, USA). Saponin derivatives were analyzed by analytical high performance liquid chromatography (HPLC) by using a Vydac C4 column (4.6 mm × 25 cm, 5 μm particles) and eluted for 30 min with a 30–48% aqueous acetonitrile linear gradient in 0.15% TFA, followed by 15 min of 48% acetonitrile in 0.15% TFA. The flow rate was 1 ml/min and the effluent was monitored by an UV detector at 214 nm.

2.1.1. Preparation of DS-1 (deacylated QS-21)

DS-1 was prepared by deacylation of QS-21 as described previously [16].

2.1.2. Preparation of deacylated crude saponins (crude deacylsaponins)

Crude *Quillaja* saponins (1.0 g) were dissolved in 50 ml water, then 0.76 g triethylamine was added to adjust a final concentration of 0.15 M triethylamine (pH 12). After the mixture was stirred at 40–50°C for 1 h, the reaction was terminated by adding acetic acid to adjust the pH to 7.0. The reaction mixture was then extracted with ethyl acetate and lyophilized. The final product was a white solid (0.82 g). The primary products of the reaction are DS-2 (deacylsaponin of QS-17 and QS-18) and DS-1 (deacylsaponin of QS-21).

2.1.3. Preparation of RDS-1 (reacylation of DS-1 and HPLC purification)

RDS-1 was prepared by mixing DS-1 (50 mg), dodecylamine (12 mg), NHS (7 mg), DCC (12 mg) and pyridine (1.0 ml). The reaction was stirred for 3 days at room temperature under N₂. Distilled water (0.1 ml) was added and the mixture was stirred for an additional 6 h to quench the reaction. Precipitated material was removed by centrifugation and then the mixture was evaporated on a rotary evaporator under vacuum to nearly dryness. The product was precipitated by adding 10 ml ethyl acetate and collected by centrifugation. The precipitate was washed twice with 2 ml ethyl acetate to afford a white solid (46 mg). ESI-MS (positive): *m/z* 1703 [*M* (DS-1 + dodecylamine) + Na]⁺,

1742 [*M* (DCC adduct of DS-1 + DCC) + Na]⁺, 1848 [*M* (diadduct of DS-1 + 2 × dodecylamine) + H]⁺, 1870 [*M* (diadduct of DS-1 + 2 × dodecylamine) + Na]⁺, 1887 [*M* (diadduct of DS-1 + dodecylamine + DCC) + H]⁺, 1909 [*M* (diadduct of DS-1 + dodecylamine + DCC) + Na]⁺.

A solution of 10 mg/ml of the above white solid in 30% aqueous acetonitrile containing 0.15% TFA was kept for 1.5–3 days at room temperature in order to allow all diadducts to decompose back to the aldehyde forms. Then 0.15 ml of the solution was injected into analytical HPLC and the peaks at 27–29.5 min and 33–38 min were collected separately (no TFA mobile phase was used). A total of five runs were carried out and the pools at 27–29.5 min and 33–38 min were combined, respectively. Acetonitrile was removed by rotary evaporation under vacuum at ~20°C and the residues were dried by lyophilization to yield 0.8 mg DCC adduct product (27–29.5 min) and 4.2 mg RDS-1 (33–38 min) respectively. RDS-1 was obtained as a white solid. ESI-MS (positive): *m/z* 1703 [*M* + Na]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 9.45 (aldehyde), 1.20 (the dodecyl CH₂ groups). The DCC adduct product was also obtained as a white solid. ESI-MS (positive): *m/z* 1742 [*M* + Na]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 9.50 (aldehyde).

2.1.4. Preparation of GPI-0100 (reacylation of crude deacylsaponins)

The mixture of above deacylsaponins (100 mg), dodecylamine (24 mg), NHS (14 mg), DCC (24 mg) and pyridine (1.5 ml) was prepared and stirred for 3 days at room temperature under N₂. Then the reaction was quenched by adding 0.1 ml distilled water and stirred for an additional 6 h. After the precipitated material was removed by centrifugation, the mixture was evaporated on a rotary evaporator under vacuum to nearly dryness. The product was precipitated by adding 10 ml ethyl acetate and collected by centrifugation. The resulting white solid was washed twice with 2 ml ethyl acetate to yield crude GPI-0100 (67 mg). ESI-MS (positive): *m/z* 1703 [*M* (DS-1 + dodecylamine) + Na]⁺, 1865 [*M* (DS-2 + dodecylamine) + Na]⁺, 1742 [*M* (DS-1 + DCC) + Na]⁺, 1904 [*M* (DS-2 + DCC) + Na]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 9.42 (aldehyde), 1.20 (the dodecyl CH₂ groups).

2.2. Immunization studies

2.2.1. Immunization protocol

Immunizations were carried out in C57BL/6 mice (female, 8–10-week-old at the time of the first immunization, 10 mice per group). The test vaccines consisted of 25 μg of the ovalbumin antigen (OVA, Grade VI, Sigma) and varying doses of the test adjuvant in a total volume of 0.2 ml phosphate buffered saline (PBS). Ovalbumin antigen (25 μg) itself without an adjuvant in a total volume of 0.2 ml PBS was used as a control and served as the “zero” adjuvant point for all groups. Vaccines were administered via the subcutaneous route and animals were immunized twice at a 2-week

interval (on days 0 and 14). Sera were collected 2 weeks after the second immunization for analysis by enzyme-linked immunosorbent assay (ELISA). Splenic mononuclear cells were collected 2 weeks after the last immunization for use as effector cells in the cytotoxic T-lymphocyte assay [9].

2.2.2. Immunological assays

Anti-OVA serum responses for IgG1 and IgG2a isotypes were determined by ELISA assays as described previously [5]. Cytotoxic T-lymphocyte responses, assayed as described previously [9], were measured as lysis of syngeneic target lymphoma cell lines (E.G7-OVA and EL4). The lytic effector cells were immunization-primed splenocytes, stimulated to mature to functional CTL by a 6-day in vitro culture with antigen (denatured OVA or mitomycin C-treated E.G7-OVA cells). Cytotoxicity was measured as lysis of ^{51}Cr -labeled target cells by splenocytes. The percent of ^{51}Cr release due to cytotoxicity was calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ where maximum release was measured after lysis of target cells with 1% NP-40 detergent and spontaneous release was measured after incubation of target cells with medium. The lysis of EL4 cells was subtracted from the lysis of E.G7-OVA cells to determine the percent antigen-specific cytotoxicity.

2.3. Hemolytic activity

An in vitro assay on red blood cells was used to screen QS-21 or analogues for hemolytic effects. Dulbecco's PBS (without calcium and magnesium) was dispensed in 100 μl aliquots to a 96-well U-bottom plate. QS-21 or analogues were serially diluted 1/2 into the buffer on the plate. Twenty-five microliters of sheep red blood cells (Biowhitaker), washed and diluted in PBS, were added to each well. The plates were incubated at room temperature for 30 min, centrifuged, 75 μl of the supernatants transferred to a flat-bottom 96-well plate and absorbance determined at 570 nm as a measure of released hemoglobin from the red cells. All analogues were assayed in triplicate. The concentration of QS-21 or analogues corresponding to 50% hemolysis was determined in each assay.

3. Results and discussion

3.1. Synthesis of saponin analogues

RDS-1 (GPI-0100 analogue of QS-21) and GPI-0100 were prepared by the literature method [17] from DS-1 and crude deacylsaponins, respectively. RDS-1 was purified by HPLC and GPI-0100 was used as a reaction mixture without further purification. Our results (based on MS analysis) showed that the reacylation reaction of DS-1 resulted in a mixture of four major products, RDS-1, a DCC adduct formed by coupling DCC with DS-1 and two diadducts (both

the carboxy group and the aldehyde group were coupled with the hydrophobic chain). The primary GPI-0100 products consist of two amides by coupling dodecylamine with DS-1 (RDS-1) and DS-2 (RDS-2) as well as two amides by coupling DCC with deacylsaponins (DCC adducts) (for chemical structures, see Fig. 1, for HPLC profiles, see Fig. 2).

3.2. Stability and solubility of GPI-0100

Although GPI-0100 was soluble in PBS at 1 mg/ml, RDS-1 was poorly soluble in PBS and it could not be filter-sterilized. The imine forms and diadducts were not stable in solution. They decomposed back to the aldehyde forms immediately in PBS solution and slowly in 30% aqueous acetonitrile solution with or without 0.15% TFA (data not shown).

3.3. Immune responses

3.3.1. Antibody responses

The adjuvant effect of QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 were evaluated in C57BL/6 mice. All compounds were evaluated over a broad range of doses together with 25 μg OVA. All analogues were shown to stimulate a similar maximum IgG1 response to optimal dose QS-21 (Fig. 3). However, the minimum dose required for maximum stimulation for all compounds was increased by several fold compared to native QS-21. There was a larger dose-shift between the analogues for IgG2a response (Fig. 4). DS-1 did not induce IgG2a at any dose evaluated (up to 240 μg). The rank order for the other compounds for minimum effective dose was QS-21 (minimum effective dose for 10-fold increase = 10 μg) \ll GPI-0100 = crude deacylsaponins (minimum dose for 10-fold increase \sim 160 μg) < RDS-1 (minimum dose for 10-fold increase \sim 240 μg). There was minimal difference for stimulation of antibody responses between GPI-0100 and crude deacylsaponins, as well as between RDS-1 and DS-1. This result indicated that addition of the lipophilic chain (dodecylamine) to the carboxyl group of the deacylsaponins does not significantly affect the adjuvant activity of these compounds for antibody response.

3.3.2. CTL response

DS-1 and RDS-1 elicit very low level CTL responses at all evaluated doses up to 240 μg . GPI-0100 and crude deacylsaponins can stimulate a very high level CTL response at high doses similar to optimal dose QS-21 (Fig. 5). Similar to antibody responses, there is no significant difference in the induction of CTL response between RDS-1 and DS-1, as well as between GPI-0100 and crude deacylsaponins. Therefore, addition of the lipophilic chain (dodecylamine) to the carboxyl group of the deacylsaponins does not significantly affect the adjuvant activity of these compounds for CTL response.

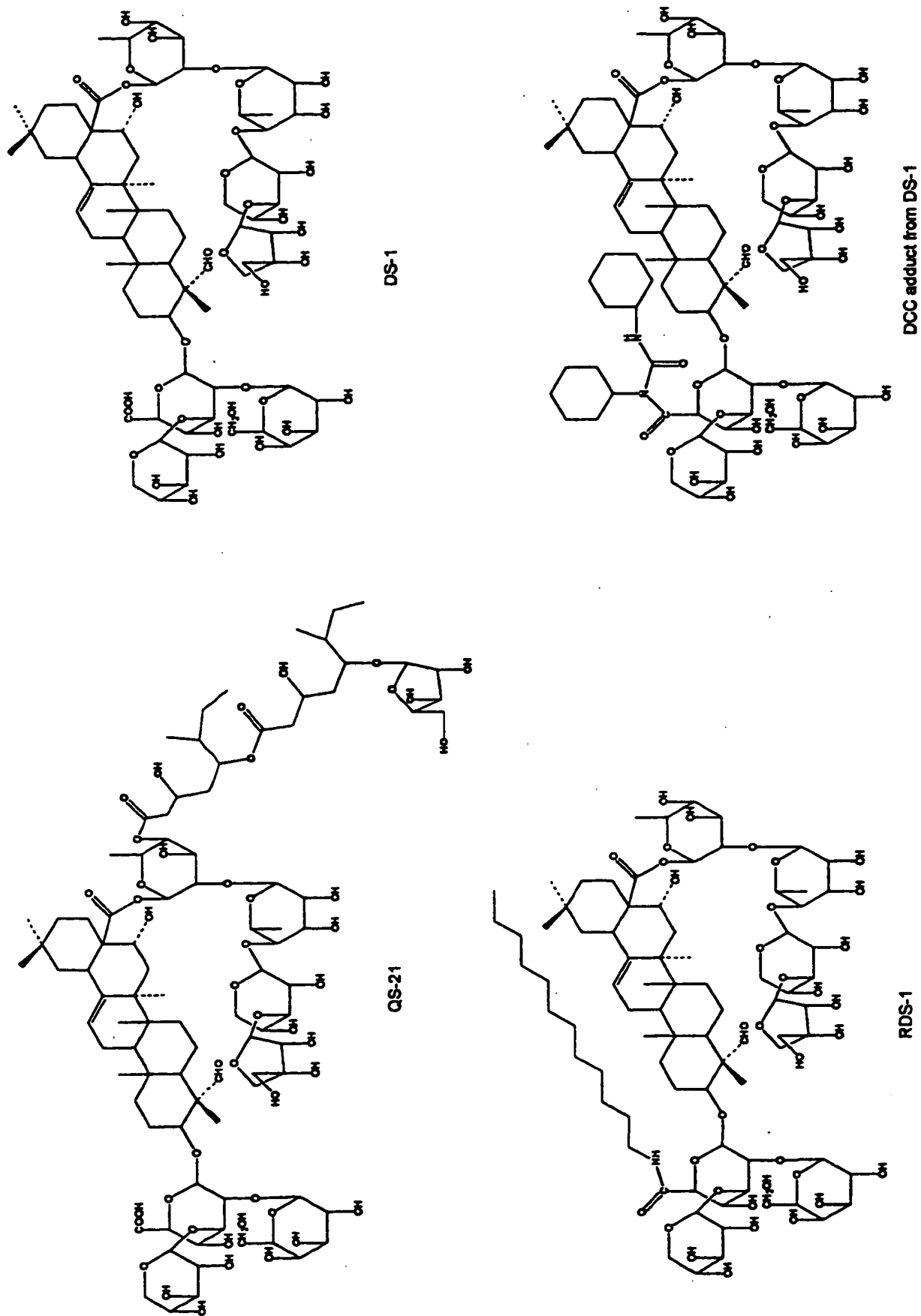


Fig. 1. Chemical structures of saponin derivatives.

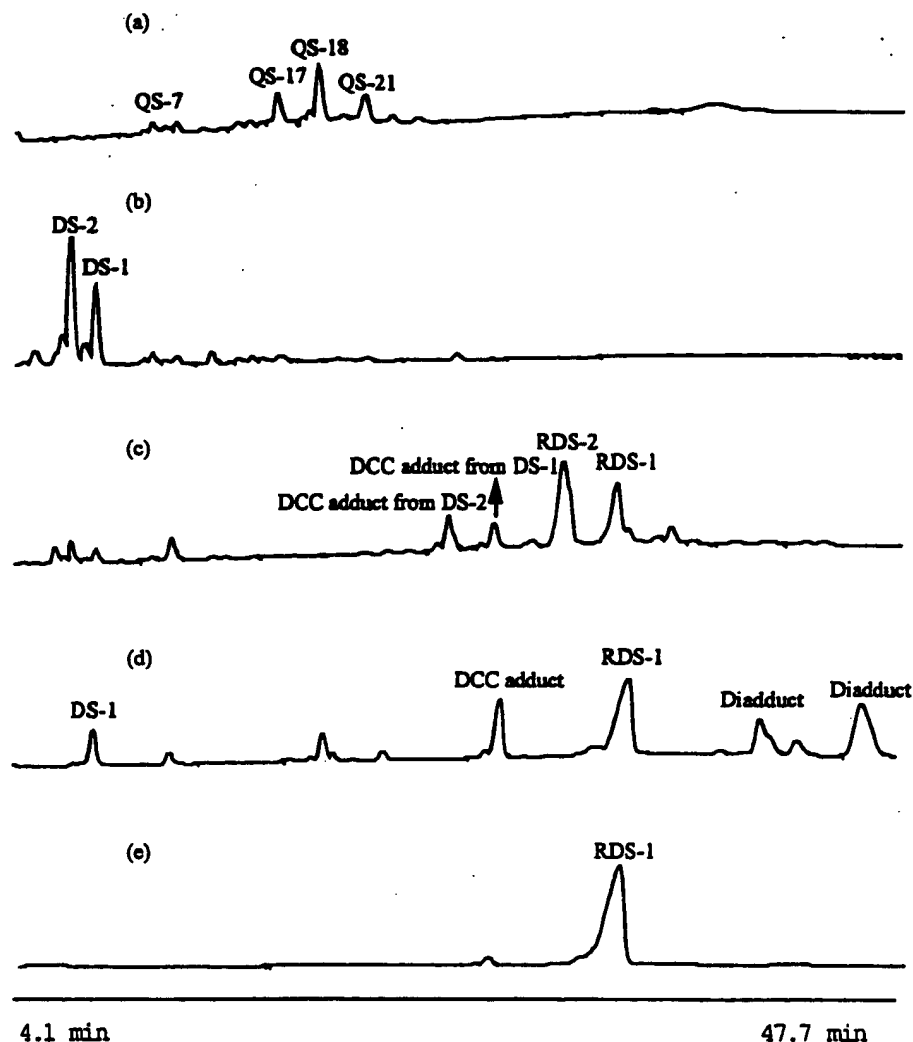


Fig. 2. HPLC profiles of (a) crude *Quillaja* saponins; (b) crude deacylsaponins; (c) GPI-0100; (d) reaction mixture of reacylation DS-1; (e) HPLC purified RDS-1.

Table 1
Fifty percent hemolysis by QS-21 or saponin derivatives ($\mu\text{g/ml}$)

QS-21	9.5
DS-1	93.0
Crude deacylsaponins	78.3
RDS-1	>500
GPI-0100	54.7

3.4. Hemolysis results

QS-21 is known to be a mild surfactant. One of the measures of this is a hemolytic assay on sheep red blood cells. This was compared between QS-21 and the analogues. RDS-1 was shown to be more than 100-fold less hemolytic than QS-21 as determined by the concentration required to produce 50% lysis. Similarly, DS-1 was shown to be less hemolytic than QS-21 by approximately 10-fold. The GPI-0100 and crude deacylsaponins were also shown to be several fold less hemolytic than QS-21 (Table 1). The

dramatic decrease in hemolytic activity of RDS-1 may be due, in part, to its poor solubility.

3.5. Summary

DS-1, crude deacylsaponins, RDS-1 (HPLC purified GPI-0100 analogue of QS-21) and GPI-0100 were prepared and their immune adjuvant activities were evaluated. Similar to previous results, QS-21 was found to stimulate IgG1, IgG2a and CTL responses at doses of 10 μg or lower. DS-1 was compared over a wide dose range to determine the effect of the acyl chain on adjuvant activity. A previous evaluation of DS-1 suggested that DS-1 was generally inactive for induction of antibody responses at doses up to 40 μg [16]. In this study, DS-1 was shown to stimulate IgG1 response at high doses, but not IgG2a. The difference may be due to the measurement of a subset of IgG in this study (IgG1 isotype) rather than the measurement of total IgG in the previous study. The lack of activity for CTL response

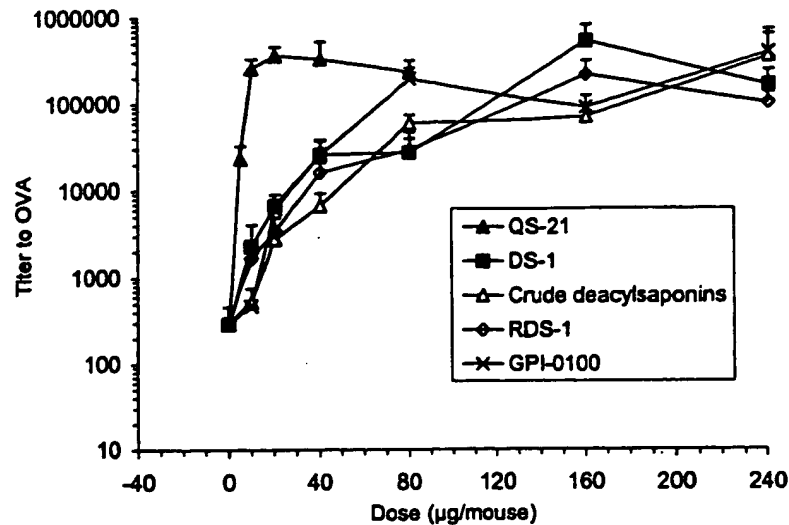


Fig. 3. Dose-response curves for QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 for stimulation of IgG1 to OVA. C57BL/6 mice (10 per group) were immunized by subcutaneous route at days 0 and 14 with 25 μ g OVA and the indicated dose of adjuvant. OVA-specific IgG1 was determined by ELISA on pooled sera collected at day 28. Two-tailed Mann–Whitney statistical analysis indicated that all evaluated QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 dose groups are statistically significant compared to the ova control group ($P < 0.05$). If the analogues groups are compared with the equivalent dose QS-21 group, all are statistically significant ($P < 0.05$) except the comparison of 80 μ g GPI-0100 group to 80 μ g QS-21 group.

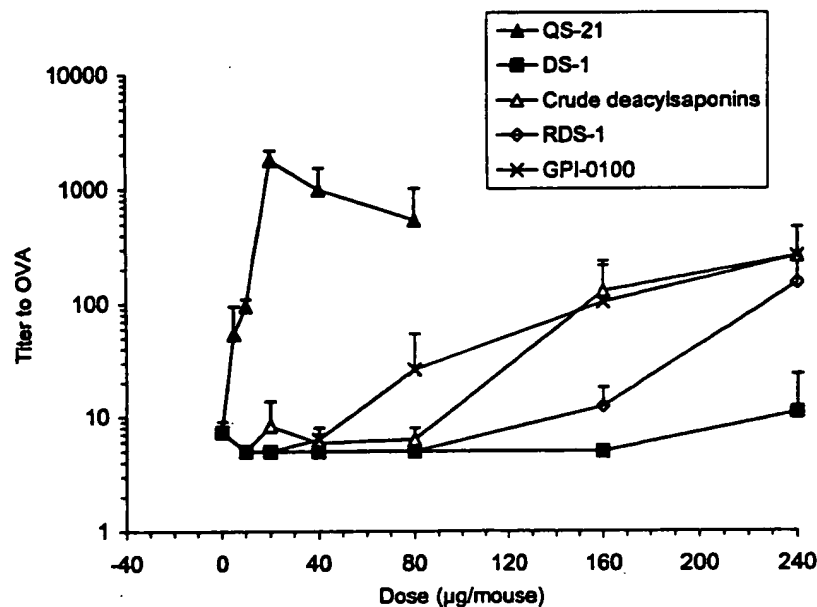


Fig. 4. Dose-response curves for effect of QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100 on IgG2a. C57BL/6 mice (10 per group) were immunized by subcutaneous route on days 0 and 14 with 25 μ g OVA and the indicated dose of adjuvant. OVA-specific IgG2a was determined by ELISA on pooled sera collected 2 weeks after the last immunization. Two-tailed Mann–Whitney statistical analysis indicated that all evaluated QS-21 dose groups are statistically significant compared to the ova control group ($P < 0.05$); all evaluated dose groups of DS-1 are not statistically significant compared to the OVA control group ($P > 0.05$); only the high dose crude deacylsaponins groups (160, 240 μ g), the high dose RDS-1 group (240 μ g) and the high dose GPI-0100 groups (160, 240 μ g) are statistically significant compared to the OVA control group ($P < 0.05$). Compared to QS-21 (the same dose group comparison), all analogue groups are statistically significant ($P < 0.05$).

(measured previously only at 10 μ g) was confirmed and found to extend to higher doses up to 240 μ g. This suggests acylation is highly critical to Th1 type responses (CTL, IgG2a), but less critical to Th2 type responses (IgG1).

We also evaluated whether reacylation of DS-1 or crude deacylsaponins would restore the adjuvant effect for IgG1,

IgG2a, or CTL associated with QS-21. Our results showed that: (1) QS-21 is a better adjuvant than RDS-1 and GPI-0100, especially for stimulation of IgG2a response; (2) there was no significant difference between GPI-0100 and crude deacylsaponins for stimulation of antibody (IgG1 and IgG2a) responses and CTL response. Similarly, there was

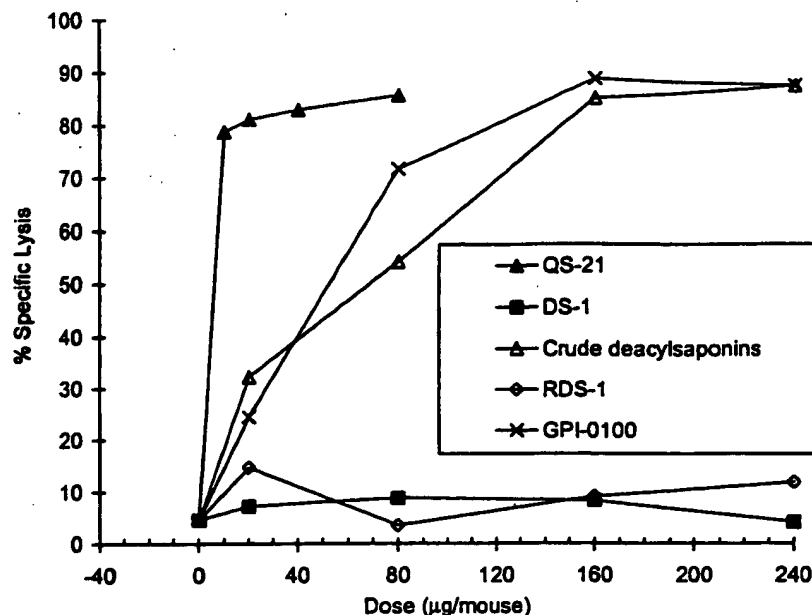


Fig. 5. Dose-response curves at 12:1 E:T ratio for production of antigen specific CTLs induced by QS-21, DS-1, crude deacylsaponins, RDS-1 and GPI-0100. C57BL/6 mice (10 per group) were immunized by subcutaneous route on days 0 and 14 with 25 μ g OVA and the indicated dose of adjuvant. The CTL response was measured on pools of splenocytes. Splenocytes were removed at day 28, expanded with antigen stimulation as described in Section 2 and used as effector cells. Lysis was measured against E.G7-OVA and EL4 cell targets.

no significant difference between the adjuvant activities of RDS-1 and DS-1. Therefore, addition of the lipophilic chain (dodecylamine) to the carboxyl group of the deacylsaponins does not significantly change the adjuvant activities. This suggests reacylation at a different site than the native compound with a synthetic fatty acid (dodecylamine) does not substantially improve the diminished adjuvant activity of the deacylated compound. We did not test other synthetic fatty acids, so cannot rule out a different result with a shorter or longer chain fatty acids. QS-7, an active native saponin, has a shorter acyl chain (C2). However, it is acylated at the original site on fucose [3]. Some residual IgG2a and CTL activity did remain in the crude deacylsaponins and GPI-0100. However, this may be due to lack of deacylation of QS-7 and other stable minor native saponins (Fig. 2). QS-7 is active in stimulating IgG2a and CTL responses [3]. It is also more stable than QS-21 under basic conditions (approximately 100-fold, Kensil, unpublished results). This is further supported by the lower activity of a reacylated saponin (RDS-1) prepared from a purified deacylsaponin (DS-1). This illustrates the importance of carrying out structure/function studies on purified natural products.

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Structure/Function Relationship in Adjuvants from *Quillaja* *saponaria* Molina

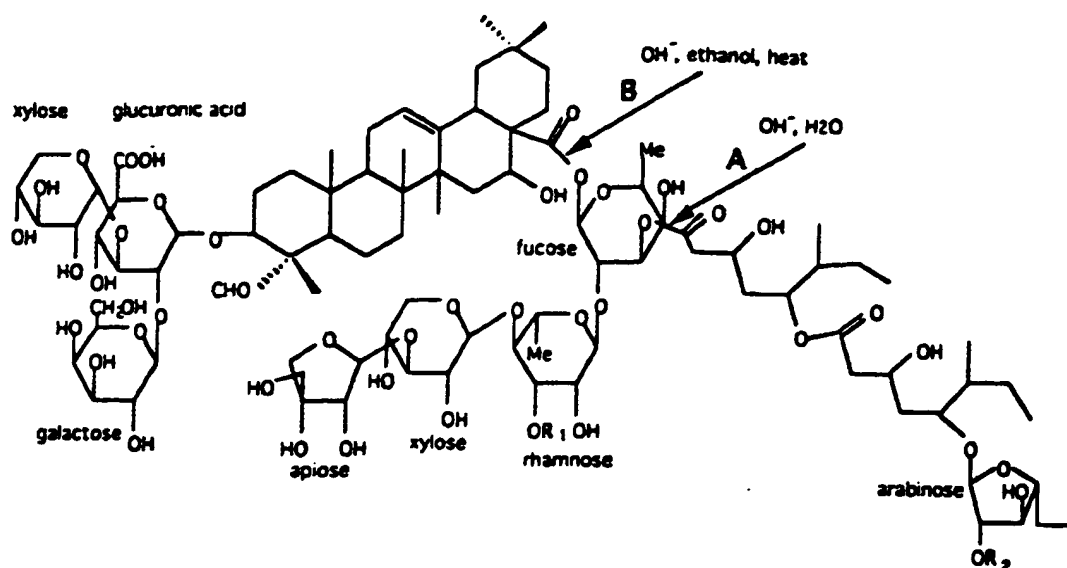
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A heterogeneous triterpene glycoside fraction from *Quillaja saponaria* Molina cortex has been utilized extensively as an immunological adjuvant, both in simple aqueous formulations (Dalsgaard 1974) and in the form of an immunostimulating complex (ISCOM) (Morein et al. 1984). Characterization of the structure of the adjuvant-active components in these extracts has been hindered by the heterogeneity of this fraction. We have purified the predominant triterpene glycoside adjuvants in crude *Quillaja* extract to near homogeneity by high-performance liquid chromatography (HPLC) (Kensil et al. 1991), allowing both structural and functional analyses. Three of these components, QS-17, QS-18, and QS-21, have been studied in more detail and form the basis of this study. Such an analysis is critical to the understanding of this important class of compounds, which have been shown to be potent adjuvants of both humoral and cell-mediated immune responses including class-I-restricted cytotoxic T lymphocytes (CTLs) to soluble proteins (Kensil et al. 1991; M.J. Newman, pers. comm.). Both types of immune responses are important to the efficacy of subunit vaccines against viral disease.

Comparative Structures of QS-17, QS-18, and QS-21

Figure 1 shows a proposed structure and the relationship among QS-17, QS-18, and QS-21. The basic structure is taken from Higuchi et al. (1988), who determined the structure of a compound (which he designated QSIII) from *Q. saponaria* that matches the carbohydrate composition and molecular weight of QS-17 reported here. We determined variations to this structure for QS-18 and QS-21 by carbohydrate composition and linkage analysis, molecular weights as determined by fast atom bombardment-mass spectroscopy (FAB-MS), and comparative analysis of common hydrolytic by-products (Fig. 1 and Table 1). The predominant changes in glycoside composition were in the terminal monosaccharides. QS-18 and QS-21 contained *t*-arabinose, whereas QS-17 contained 2-arabinose. QS-17 contained *t*-rhamnose (not present in QS-18 and QS-21), suggesting that *t*-rhamnose was linked to 2-arabinose in QS-17, whereas arabinose was a terminal residue in QS-18 and QS-21. In addition, QS-17 and QS-18 contained 3,4-rhamnose and *t*-glucose, whereas QS-21 contained 4-rhamnose and no glucose. This was indicative of glucose substitution at the 3 position of 3,4 rhamnose in QS-17 and QS-18.

Differences in the molecular weights, determined by FAB-MS, were consistent with these proposed structures. Further support was provided by comparison of hydrolytic by-products. Higuchi has demonstrated that mild alkaline hydrolysis of QSIII results in cleavage at the ester bond linking the fatty acid moiety to fucose. Cleavage of QS-17, QS-18, and QS-21 at this site should yield a triterpene glycoside fragment (A) that is identical for QS-17 and QS-18 and is more hydrophobic for QS-21 (due to absence of



	R ₁	R ₂	m/z	Reverse Phase Retention Time(min)	
				Fragment A	Fragment B
QS17	glucose	rhamnose	2321	8.0	26.7
QS18	glucose	H	2174	8.0	26.4
QS21	H	H	2012	9.3	25.6

Figure 1

Proposed structure of QS-17, QS-18, and QS-21. Structure of QS-17 was taken from QSIII (Higuchi et al. 1988). Fragments A and B from each compound were generated by the indicated hydrolysis conditions, encompass the triterpene glycoside portion of the molecules, and were analyzed by reversed-phase HPLC.

Table 1
Carbohydrate Analysis of Purified *Quillaja saponaria* Adjuvants

	QS-17			QS-18			QS-21		
	AA ^a	TMC ^b	linkage	AA	TMC	linkage	AA	TMC	linkage
Rhamnose	184	2.34	T ^c 3,4	132	1.15	3,4 ^d	132	1.27	4
Fucose	78	0.96	2	96	0.88	2	100	0.91	2
Arabinose	65	0.98	2	80	0.74	T	71	0.77	T
Xylose	82	1.33	T 3	118	1.34	T 3	114	1.44	T 3
Galactose	69	1.00	T	88	1.00	T	88	1.00	T
Glucose	86	1.23	T	89	1.16	T	-	-	-
Glucuronic acid	n.t. ^e	0.64	2,3	n.t.	0.72	2,3	n.t.	0.74	2,3
Apiose	24.5 ^f	n.t.	T	25.7	n.t.	T	20.0	n.t.	T

^aAlditol acetate (μg/mg compound).

^bTrimethylsilylated methyl glycosides (mole monosaccharide/mole galactose).

^cTerminal glycosyl residue.

^dTrace amounts of T-rhamnose also detected.

^en.t. indicates not tested.

^fPoor recovery as alditol acetates.

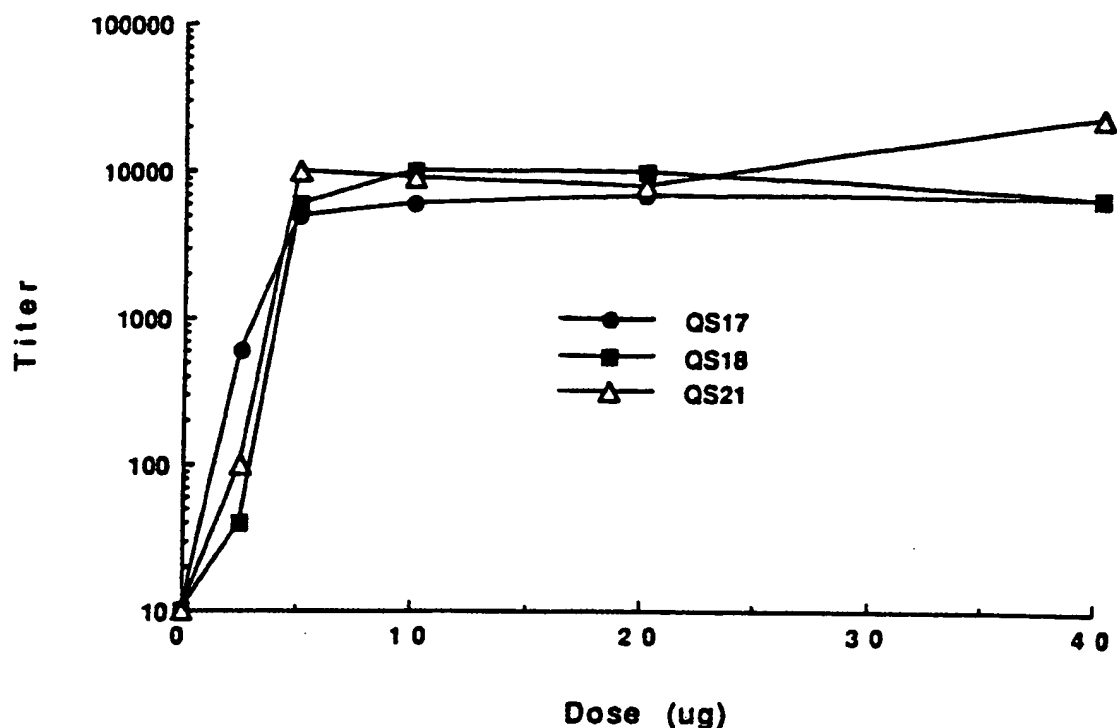


Figure 2

Dose response study of QS-17, QS-18, and QS-21 for antibody stimulation. CD-1 mice (five per group) were immunized intradermally with 5 μ g of BSA and the indicated dose of adjuvant at day 0 and day 14. Sera were analyzed by EIA on BSA-coated plates on day 21.

glucose); this was confirmed experimentally by reversed-phase HPLC retention times of the fragments from these compounds (Fig. 1). These compounds were hydrolyzed under more severe conditions to cleave the ester bond linking fucose to the quillaic acid backbone; the limiting triterpene glycoside fragment (B) resulting from this cleavage should be identical for all three compounds. This was confirmed by HPLC analysis (Fig. 1).

All three compounds, QS-17, QS-18, and QS-21, had been shown to augment humoral immune responses in mice with similar dose response curves (Fig. 2). Hence, it would appear that the terminal residues rhamnose and glucose are not critical to this facet of the adjuvant function of these compounds.

Influence of Structural Modifications on Adjuvant Activity

Modification of these compounds was carried out to determine the effect on antibody stimulation. QS-18 was modified by periodate oxidation, which preferentially generates aldehydes from *cis* vicinal hydroxyl groups. Hence, *t*-galactose and *t*-apiose were likely targets of this reagent, allowing assessment of the importance of these monosaccharides to adjuvant function. The periodate-oxidized QS-18 was tested for augmentation of antibody response to bovine serum albumin (BSA) in mice (Fig. 3A). Periodate oxidation of QS-18 eliminated adjuvant activity. Hence, either galactose or apiose (or both), which are monosaccharides common to all known adjuvant-active compounds from *Q. saponaria* for which carbohydrate composition data are available, appears to be essential for antibody stimulation.

Hydrolytic by-products of QS-18 and QS-21 (equivalent to fragment A of Fig. 1) were prepared to determine the effect of removal of the fatty acids and arabinose on adjuvant

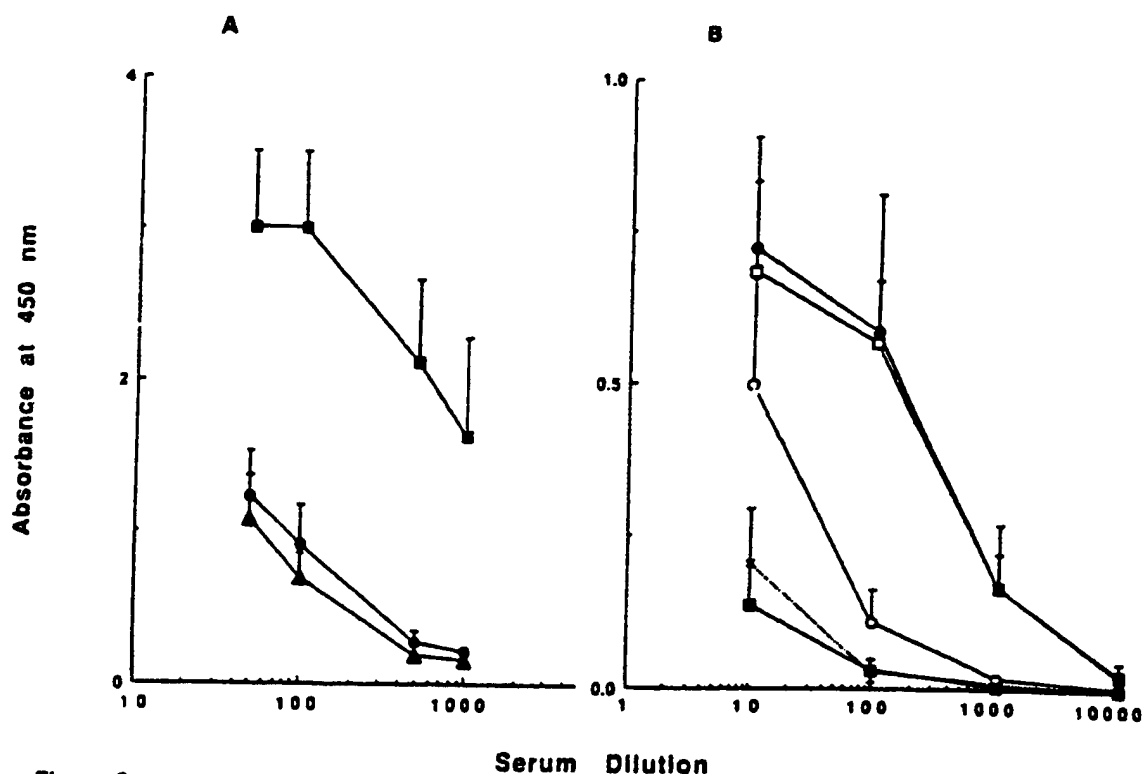


Figure 3

(A) Comparison of adjuvant effect of QS-18 and periodate-oxidized QS-18. (Closed circle) No adjuvant; (closed triangle) periodate oxidized QS-18; (closed box) QS-18. (B) Comparison of adjuvant effect of QS-18 and QS-21 with the respective fragment A. (Dotted line) No adjuvant; (open box) QS-18; (closed box) fragment A of QS-18; (closed circle) QS-21; (open circle) fragment A of QS-21. CD-1 mice (3-4 per group) were immunized intradermally with 10 μ g BSA and 10 μ g of the indicated adjuvant. Sera were analyzed by EIA on BSA-coated plates on day 14.

function. QS-18 and QS-21 were treated by mild alkaline hydrolysis to generate their respective fragment A. These fragments were substantially more hydrophilic than the original compounds due to loss of the fatty acids and arabinose. These fragments were tested in mice for induction of antibody to BSA (Fig. 3B). The fatty-acid-free fragments induced a substantially lower antibody response than the formulations containing the intact adjuvants. We had previously suggested that a close association of antigen and *Q. saponaria* adjuvants could be important for optimum immune response (Kensil et al. 1991). We have observed that adjuvant injected in a site different from that of antigen is ineffective. QS-18 and QS-21 have been shown to bind to BSA (data not shown). One possible mechanism for this decreased antibody stimulation by the fatty-acid-free glycosides is that the binding to the antigen through hydrophobic interactions is reduced or eliminated due to the absence of the fatty acid. However, it should be noted that the loss of adjuvant function of periodate-oxidized QS-18 could not be attributed to change in hydrophobicity of the compound because the oxidation had only a minimal effect on hydrophobicity.

To investigate the importance of the close association further, we have covalently coupled QS-21 to a protein antigen, hen egg lysozyme. Lysozyme was chosen for this experiment because in general it is poorly immunogenic, and being a hydrophilic protein, it is unlikely to bind to QS-21 through hydrophobic interactions. A conjugate (1:1 molar ratio of QS-21:lysozyme, prepared by coupling the carboxylic acid on QS-21 glucuronic acid to protein amino groups with carbodiimide chemistry) was tested in C57BL/6 mice (Fig. 4). QS-21 failed to produce detectable anti-lysozyme antibody titers in mice im-

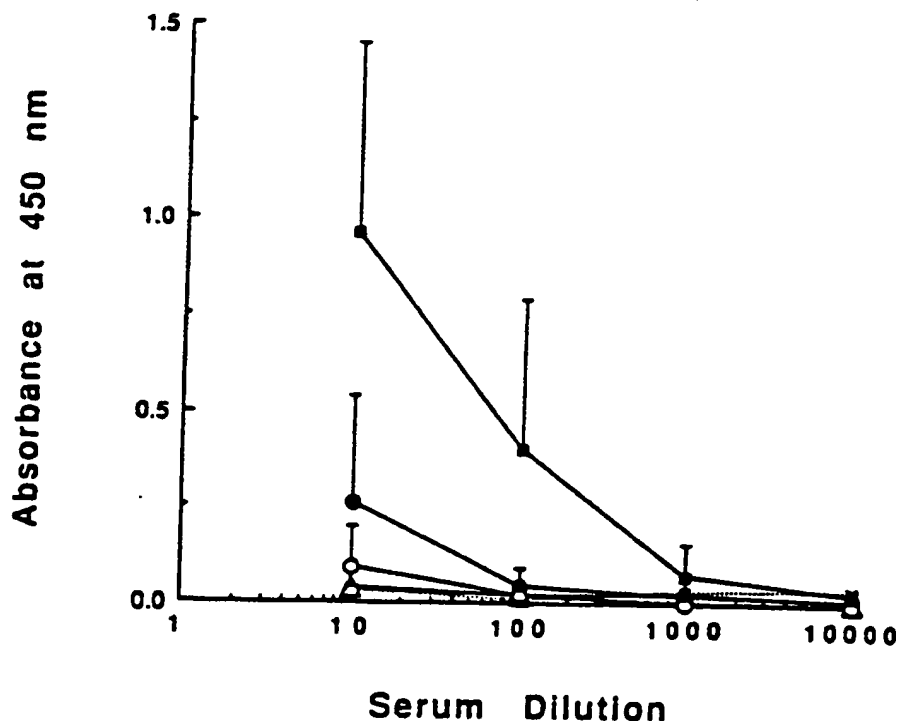


Figure 4

Comparison of immune response of an antigen-QS-21 conjugate compared to unconjugated antigen and QS-21. C57BL/6 mice (ten per group) were immunized intradermally at 1 and 14 days with lysozyme or lysozyme-QS-21 conjugates. Sera were analyzed by EIA on lysozyme-coated plates on day 21. (Dotted line) 10 µg lysozyme; (open triangle) 10 µg lysozyme/1.6 µg QS-21 (noncovalent); (closed circle) 10 µg lysozyme/1.6 µg QS-21 (covalent); (open circle) 10 µg lysozyme/10 µg QS-21 (noncovalent); (closed box) 10 µg lysozyme/1.6 µg QS-21 (covalent) + 10 µg QS-21 (noncovalent).

munized twice with 1.6 µg of free QS-21 mixed with lysozyme and only increased titers slightly in mice immunized with 10 µg of free QS-21, a result that we think may be due to lack of QS-21 binding to lysozyme. Mice immunized with an equimolar conjugate (amount of bound QS-21 = 1.6 µg) produced an immune response to lysozyme that exceeded that even of mice receiving 10 µg of QS-21 in noncovalent form. Addition of 10 µg of free QS-21 to the conjugate induced the highest responses, suggesting that the covalently attached QS-21 served as a association site for an additional one to two molecules of QS-21 to lysozyme.

SUMMARY

We have shown that galactose and apiose, monosaccharides common to the structures of QS-17, QS-18, and QS-21, are critical to their function in stimulation of antibody. In contrast, *D*-rhamnose and *D*-glucose do not seem to be important because these monosaccharides are the primary points of deviation among these three compounds, which are very similar in antibody stimulation. The fatty acid region also appears to play an important role. In addition, the data on conjugation of QS-21 to antigen suggested that close association of antigen and this adjuvant is important. At present, little is known about the mechanism of this important class of adjuvants. Future studies will entail determination of the cellular site of action and development of in vitro assays to assess the function of this class of important compounds.

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SEPARATION AND CHARACTERIZATION OF SAPONINS WITH ADJUVANT ACTIVITY FROM *Guillaja saponaria* MOLINA CORTEX

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Saponins were purified from *Guillaja saponaria* Molina bark by silica and reverse phase chromatography. The resulting purified saponins were tested for adjuvant activity in mice. Several distinct saponins, designated QS-7, QS-17, QS-18, and QS-21, were demonstrated to boost antibody levels by 100-fold or more when used in mouse immunizations with the Ag BSA and beef liver cytochrome *b₅*. These purified saponins increased titers in all major IgG subclasses. To determine optimal dose in mice for adjuvant response, QS-7 and QS-21 were tested in a dose-response study in intradermal immunization with BSA in mice; for both of these purified saponins, adjuvant response (determined by stimulation of ELISA titers to BSA) neared maximum at doses of 5 µg and was shown to plateau up to the highest dose tested, 80 µg. These purified saponins vary considerably in their toxicity, as assessed by lethality in mice; the main component, QS-18, being the most toxic. Saponins QS-7 and QS-21 showed no or very low toxicity in mice, respectively. None of these saponins stimulated production of reagenic antibodies. The monosaccharide composition of these saponins showed similar but distinct compositions with all four containing fucose, xylose, galactose, and glucuronic acid. Predominant differences were observed in the quantities of rhamnose, arabinose, and glucose. Monomer m.w. (determined by size exclusion HPLC) were determined to range from 1800 to 2200.

Formulation of effective vaccines requires not only the appropriate Ag, but also the appropriate adjuvant to optimize protective humoral and cell-mediated immune responses. The use of the same Ag with different adjuvants has been shown to elicit significantly different responses from the immune system. For example, comparison of immunization of mice with killed schistosomula from *Schistosoma mansoni* with the adjuvants bacillus Calmette-Guérin, pertussis, *Coryne bacterium parvum*, tetanus toxoid, *Escherichia coli* LPS, yeast glucan, aluminum hydroxide, and saponin showed that only the ani-

mals immunized with bacillus Calmette-Guérin or saponin were protected from challenge (1) despite the demonstration of significant humoral immunity by some of the ineffective adjuvants. In effect, Allison et al. have noted that adjuvants such as aluminum hydroxide and mineral oil produce primarily humoral immunity whereas adjuvants such as muramyl dipeptide are able to induce cell-mediated immunity as well as differences in the isotype of the antibodies elicited (2). A further consideration, in addition to the efficacy of the adjuvant for eliciting a protective immune response, is the issue of toxicity of the adjuvant. CFA, which is used widely in research vaccines, produces excellent humoral and cell-mediated immunity, but is unsuitable for use in human and veterinary vaccines because of the toxic side effects (3). Similarly LPS, which is also a strong adjuvant, is highly toxic (reviewed in Reference 4). Hence, there is a need for identification of adjuvants that are both safe and efficacious.

One such potential adjuvant system is a class of compounds extracted from plant sources, termed collectively as saponins because of the detergent properties associated with them. The detergent properties of saponins are caused by their amphipathic nature; they consist of a hydrophilic carbohydrate moiety and a hydrophobic steroid or triterpene moiety. The adjuvant effect of saponins was noted in 1951 by Espinet (5) who utilized a crude saponin mixture to increase the immune response to foot-and-mouth disease vaccine. Extracts of the bark of a South American tree, *Guillaja saponaria* Molina, have been shown to be potent adjuvants (6-8). Further studies by Dalsgaard showed that adjuvant activity in these extracts resides in the saponin fraction, which has been characterized as a mixture of triterpene glycosides (7). Crude preparations of *Guillaja* saponins have been used to boost the response to BSA (7), keyhole limpet hemocyanin (9), SRBC (8), as well as aluminum hydroxide-based vaccines (9, 10). In addition, partially purified *Guillaja* saponins have been reported to associate with hydrophobic or amphipathic proteins and lipids to form detergent/lipid/saponin complexes termed ISCOM* (11); these structures are typically prepared by solubilizing the Ag with non-ionic detergents and then exchanging the non-ionic for the saponin detergent by centrifugation through sucrose gradients containing saponins at a concentration higher than their critical micellar concentration. ISCOM, which have been prepared from surface Ag isolated from influenza virus, measles, toxoplasma, feline leukemia virus, EBV, and HIV-1 (11-13) induce

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* Abbreviations used in this paper: ISCOM, immunostimulating complexes; MDP, muramyl dipeptide; MPL, monophosphoryl lipid A; TDM, trehalose dimycolate; TFA, trifluoroacetic acid; I.D., inside diameter.

serum antibody titers that are approximately 10-fold higher than immunization with protein micelles alone.

In addition to the potent adjuvant activity, the saponin fraction from *Quillaja* bark has strong hemolytic activity (7). This hemolytic activity has been suggested to be caused by the intercalation of saponins into cholesterol-containing membranes to form holes of approximately 80 Å, which can be observed with negative staining electron microscopy (14–16).

Despite the potential use of *Quillaja* saponins as adjuvants, their application has been limited because of the undesirable side effects of the commercially available preparations that are partially purified mixtures of saponins and other components (17–20). The adjuvant-active saponins have not been characterized because of the difficulty in purifying the active components to homogeneity. An adjuvant-active fraction was prepared from an aqueous extract of *Q. saponaria* bark by Dalsgaard (7) by using dialysis, anion exchange, and gel filtration chromatography in aqueous buffers; this fraction (designated Guil-A) was reported to be a single band by TLC on silica gel plates. However, we have found that this fraction is still a heterogeneous saponin mixture that can be resolved into multiple glycoside fractions by reverse phase HPLC. Higuchi et al. (21) have recently substantially purified a saponin from a methanolic extract of *Quillaja* bark and have characterized the glycoside moiety; however, this purified saponin was not tested for adjuvant effect. Hence, at present, there is no information on which components of the saponin fraction from *Quillaja* bark possess adjuvant activity. In this paper, we report a separation procedure for saponins extracted from the cortex of *Q. saponaria* Molina. Identification of distinct saponin components with adjuvant activity and no apparent lethality in mice in an adjuvant-active dose range. Identification of an adjuvant-saponin that is lethal at a lower dose than the original aqueous extract, and preliminary chemical characterization of these fractions.

MATERIALS AND METHODS

Purification of saponins. Coarsely chopped *Q. saponaria* bark (approximately 1 cm square, obtained from Hauser Chemicals, Boulder, CO) was stirred with 10 ml of water/g of bark at room temperature for 1 h. The extract was centrifuged and the supernatant containing the solubilized saponins was saved. The extraction step was repeated on the bark pellet and the two supernatants were pooled. To remove nonsaponin components, the supernatant pool was lyophilized, redissolved in 40 mM acetic acid in water at a concentration of 250 mg/ml (w/v) and either chromatographed through Sephadex G-50 (medium, Pharmacia, Piscataway, NJ) in 40 mM acetic acid with the hemolytic activity localized in the void volume fraction, or dialyzed against 40 mM acetic acid with the hemolytic activity retained by the dialysis membrane.

The hemolytic fraction was lyophilized and redissolved at a concentration of 200 mg/ml in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v); 1 g of this fraction was applied to Silica Lichroprep (E. M. Science, Gibbstown, NJ; 40 to 63 µm particle size, 2.5 cm I.D. × 20 cm height) and eluted isocratically in the solvent used to solubilize the saponins. The elution of saponins was monitored by carbohydrate assay (22). Fractions containing the saponins of interest were identified by reverse phase TLC with visualization with Bial's reagent (Sigma, St. Louis, MO) pooled individually, and rotavapped to dryness. The fractions from the silica chromatography were then redissolved in 40 mM acetic acid in 50% methanol and loaded on a semipreparative HPLC column (Vydac C₈, 5 µm particle size, 3000 nm pore size, 10 mm I.D. × 25 cm length). Saponin peaks, detected by absorbance at 214 nm, were eluted by using a methanol gradient at a flow rate of 4 ml/min, and individually rotavapped to dryness. Purity of saponins was assessed by analytic HPLC (Vydac C₈, 5 µm particle size, 3000 nm pore size, 4.6 mm I.D. × 25 cm length) with a gradient of 0.1% TFA in acetonitrile.

Immunologic procedures. CD-1 mice (8 to 10 wk of age) were immunized intradermally with a total volume of 0.2 ml injected at two sites per mouse. Each sample was tested in a group of five mice. The buffer used for all immunizations was PBS. The following proteins were used as Ag: BSA (Sigma) and purified cytochrome b₅ from beef liver, kindly provided by Dr. Philipp Strittmatter (University of Connecticut Health Center, Farmington, CT). CFA and IFA were obtained from Difco (Detroit, MI). MPL and TDM were obtained from Ribi Immunochemicals (Hamilton, MT). Squalene and Tween-20 were obtained from Sigma. Superfos Guil-A, a crudely enriched saponin preparation, and Alhydrogel (2% aluminum hydroxide) were obtained from Accurate Sciences, Westbury, NY.

The toxicities of Guil-A and purified saponins GS-7, 18, and 21, were tested in CD-1 mice by following procedures similar to those described above for immunizations. Varying doses of these compounds dissolved in sterile PBS were injected intradermally in mice. The mice were monitored for 72 h after injections and the results expressed in number of deaths per group.

Ag-specific antibody response was determined by ELISA. Immulon II plates were coated overnight at 4°C with 100 µl/well of coating solution, consisting of 10 µg/ml of the Ag in PBS. Plates were then washed twice with PBS and blocked in 10% normal goat serum (Hazelton, Rockville, MD) in PBS (150 µl/well for 1 h at room temperature). Plates were washed twice with 0.05% Tween 20 (Sigma) in water. Mouse serum was serially diluted 1/10 in 10% normal goat serum in PBS; 100 µl of each dilution was incubated on the plate for 1 h at room temperature. All dilutions were tested in duplicate on both Ag-coated and noncoated control wells. Plates were washed twice with 0.05% Tween 20. Goat anti-mouse IgG-horseradish peroxidase conjugate (H and L chain specific; Boehringer-Mannheim Indianapolis, IN), diluted in 10% normal goat serum in PBS, was incubated on the plate (100 µl/well for 30 min at room temperature). The plates were washed four times with 0.05% Tween 20 and then with water two times. The substrate for the reaction was tetramethylbenzidine (23). Titers were determined from the dilution resulting in an absorbance of 0.5. Relative titers of specific antibody isotypes were determined by titration of sera pools (prepared with equivalent ratios of individual mouse serum samples in a group) on Ag-coated plates with the use of goat anti-mouse alkaline phosphatase conjugates specific for IgM, IgG3, IgG1, IgG2a, and IgG2b, respectively (Southern Biotechnology Associates, Birmingham, AL) and a goat anti-mouse IgE-horseradish peroxidase conjugate (Nordic, El Toro, CA).

Hemolytic activity. Serial 1/2 dilutions of saponin in PBS were made in a round bottom microtiter plate. The final volume in each well was 100 µl. SRBC (40% sheep blood and 60% Alsever's solution; Whittaker Bioproducts, Walkersville, MD) were washed three times by low speed centrifugation of the blood followed by resuspension of the red cell pellet in PBS to the original volume. The red cell pellet was diluted to 2.5 × the original volume and then used in the hemolysis assay. Twenty-five microliters of the resuspended cells were added to each well in the microtiter plate and mixed by pipetting. After incubation at room temperature for 30 min, the plates were spun at 1000 rpm for 5 min in a Sorvall RT6000 in an H-1000 rotor to sediment unhemolyzed cells. Fifty microliters of the supernatant from each well were transferred to the same well of a flat bottom microtiter plate. Absorbance caused by released hemoglobin was determined at 570 nm with a Dynatech microtiter plate reader.

Carbohydrate analysis. Relative carbohydrate concentration was determined by the anthrone method of Scott and Melvin (22). The standard for the assay was glucose. Analysis of carbohydrate composition as trimethylglucosides was carried out under contract by the Complex Carbohydrate Corporation (Athens, GA).

Monomer size of saponins. Monomer size of the saponins was determined by HPLC gel permeation chromatography on a Zorbax PSM 60 Si column (6.2 mm I.D. × 25 cm height). Ginsenoside Rb₁ (m.w. = 1109; Waco Pure Chemicals, Dallas, TX) and 18-β-glycyrrhetic acid (m.w. = 471; Fluka Chemicals, Everett, WA) were used as m.w. standards. Saponins and standards were solubilized in methanol at a concentration of 1 mg/ml. Twenty microliters were injected on the column and eluted in methanol at a flow rate of 1.0 ml/min. Absorbance at 214 nm was used to monitor the column.

RESULTS

Isolation and characterization of saponin adjuvants. Approximately 20 to 25% of the dry weight of *Q. saponaria* Molina bark is extractable in water. Dialysis of the aqueous extract resulted in retention of approximately 24% of the dry weight and 95% of the hemolytic activity of the extract, indicating that saponins present in the

aqueous bark extract were retained by a dialysis membrane of 12,000 m.w. cutoff. Similar recoveries were achieved by chromatography of the aqueous extract on Sephadex G-50, with the saponin fraction localized in the void volume; reverse phase TLC showed that the identical components were isolated (not shown).

With the use of reverse phase HPLC, an unprocessed extract of *Q. saponaria* bark was shown to be a highly complex mixture. Treatment of this aqueous extract by ultrafiltration through a membrane with 10,000 m.w. cutoff removed almost all hydrophilic peaks from the retentate although multiple hydrophobic components were still present (Fig. 1A). Analysis of Quil-A, a commercial saponin that is commonly used in adjuvant studies, showed that this product contains all the peaks present in the ultrafiltrated aqueous bark extract shown in Figure 1A.

Significant resolution of the saponin peaks in the ultrafiltration retentate was achieved by using a shallow

gradient of methanol or acetonitrile on Vydac C₄ as described in *Materials and Methods* (Fig. 1A). All major peaks in this retentate fraction were reactive with anthrone, indicating the presence of carbohydrate, and caused foaminess in aqueous solution, indicating that they were saponin in nature. Different bark samples yielded qualitatively a similar pattern of peaks with the same retention times. However, some quantitative differences were observed between different bark samples, apparently as a result of differences between the bark samples because extractions from the same sample of bark yield consistent results. The saponin peaks isolated by HPLC were tested for adjuvant activity by using BSA as the test Ag. Adjuvant-active components were identified in 10 of the peaks tested including the major peaks (7, 17, 18, and 21) (data not shown). These peaks, particularly peak 18, predominate in most samples of bark or commercial *Quillaja* saponins tested.

The major saponin peaks, purified as described in *Materials and Methods*, were further characterized for adjuvant activity as well as for physical and chemical properties. The purity of these samples is shown in Figure 1. The fractions, designated as saponins QS-7, 17, 18, and 21, with QS denoting the source to be *Q. saponaria*, are significantly pure in comparison with the starting extract, although several minor contaminants are evident in some fractions (Fig. 1 B to E).

Effect of dose on adjuvant effect in mice. To establish the range of effectiveness for purified saponins, dose response curves were carried out for two of the saponins, QS-7 and QS-21 (Fig. 2). These saponins were chosen because they represented the most hydrophilic (QS-7) and hydrophobic (QS-21) of the four saponins purified in this study. Hydrophobicity was assumed to be related to the retention time on reverse phase HPLC with the use of a hydrophobic resin. CD-1 mice were immunized intradermally twice with BSA plus the indicated dose of saponin at 2-wk intervals. Sera was analyzed for anti-BSA IgG by ELISA 1 wk after the second immunization. Anti-BSA IgG titers were considerably augmented by doses of saponin as low as 5 μ g for both QS-7 and QS-21. The immune responses obtained with QS-7 and QS-21 were similar, reaching a plateau at doses between 10 and 80 μ g. No significant differences were observed between QS-7 and QS-21.

Adjuvant activity of purified saponins and research adjuvants. The purified *Quillaja* saponins (QS-7, 17, 18 and 21) were compared for effectiveness as adjuvants with various research adjuvants, such as aluminum hydroxide, CFA, and IFA, and a mixture of MPL and TDM. Saponins were used at a dose of 20 μ g, an amount that falls in the plateau of maximum adjuvant effect observed with QS-7 and QS-21. Two immunizations with 10 μ g of Ag cytochrome b₅ plus QS-7, 17, 18, or 21 in PBS resulted in an increase of approximately 10^3 in Ag-specific IgG ELISA titers when compared to a control group that received Ag alone. The titers observed in the groups receiving purified saponins were similar to those induced by the MPL/TDM mixture and CFA and IFA. However, purified saponins induced a higher response than aluminum hydroxide (Fig. 3).

Isotype of antibodies augmented by saponins. Adjuvants that augment similar IgG titers may differ considerably in boosting various IgG subclasses. Therefore, the

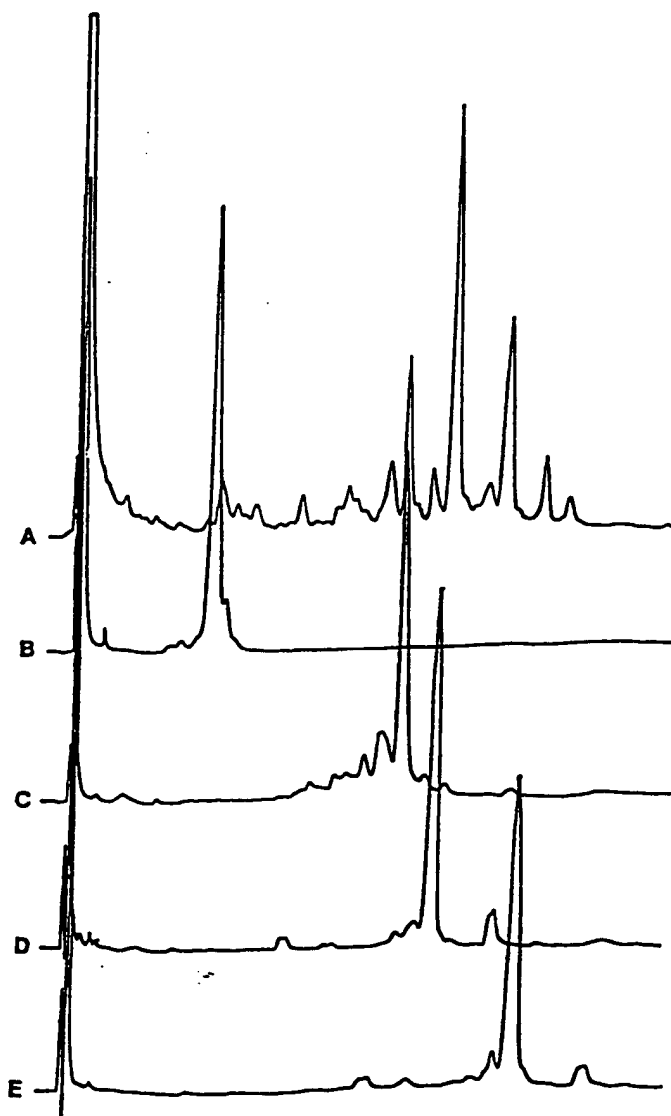


Figure 1. HPLC (Vydac C₄, 4.6 mm \times 25 cm, 5 μ m particle size, 3000 nm pore size) of an aqueous bark extract treated by ultrafiltration (A), saponin QS-7 (B), saponin QS-17 (C), saponin QS-18 (D), and saponin QS-21 (E). Gradient was 30 to 40% 0.1% TFA/acetonitrile/30 min, 40%/15 min at a flow rate of 1 ml/min. A total of 100 μ g of purified saponin or 200 μ g bark extract (dry weight) was used per injection.

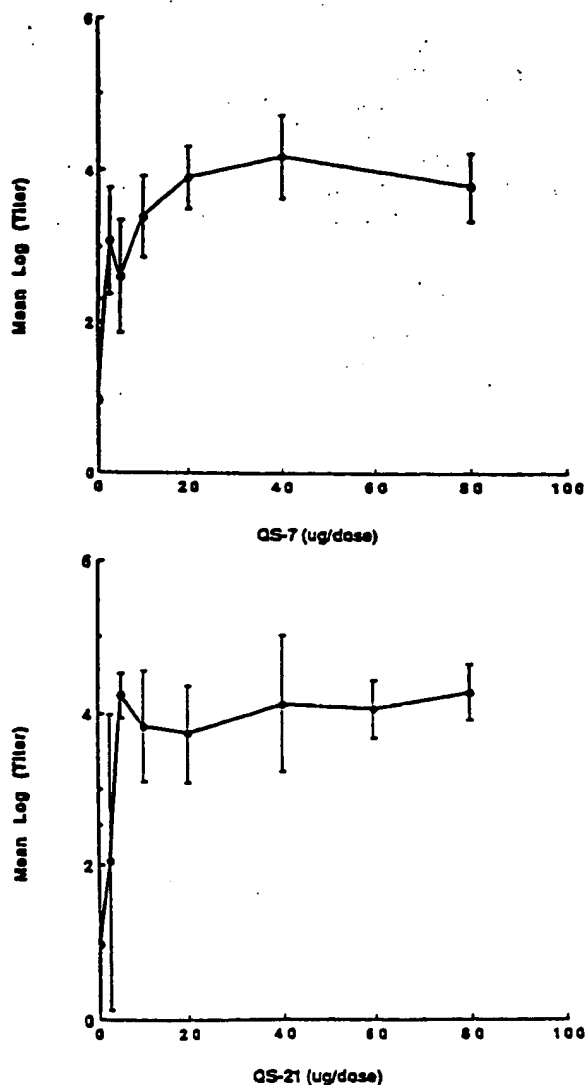


Figure 2. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 5 μ g BSA and the indicated dose of QS-7 and QS-21. Results are expressed as means \pm SD.

IgG subclass distribution of the IgG for the immunization experiment described in Figure 3 was determined. After two intradermal immunizations with cytochrome b_5 and saponins QS-7, 17, 18, and 21, antibodies were found in the three major IgG subclasses G1, G2_a, and G2_b (Table I). With saponin fractions QS-17, 18 and 21, IgG2a antibodies predominated. In contrast, antibodies induced by Ag in PBS or on aluminum hydroxide were predominantly IgG1. CFA and MPL/TDM adjuvant augmented the production of isotypes IgG1, IgG2a, and IgG2b whereas IFA induced isotypes IgG1 and some IgG2b. In contrast to previous reports with the use of crude saponin preparations from *Q. saponaria* (2), no IgE antibodies were elicited by any of the purified saponins described here. Regainic antibodies were not detectable at a 1/10 dilution for any of the adjuvants tested. The dose dependence of isotype distribution was not determined.

Purified saponins yielded consistent results in adjuvant effect. Five preparations of QS-21 that had been purified from different sources of *Q. saponaria* Molina bark were tested concurrently in an immunization study with BSA in mice; the mean and SD of the log₁₀ ELISA titer of the five groups receiving three injections of 15 μ g of QS-21

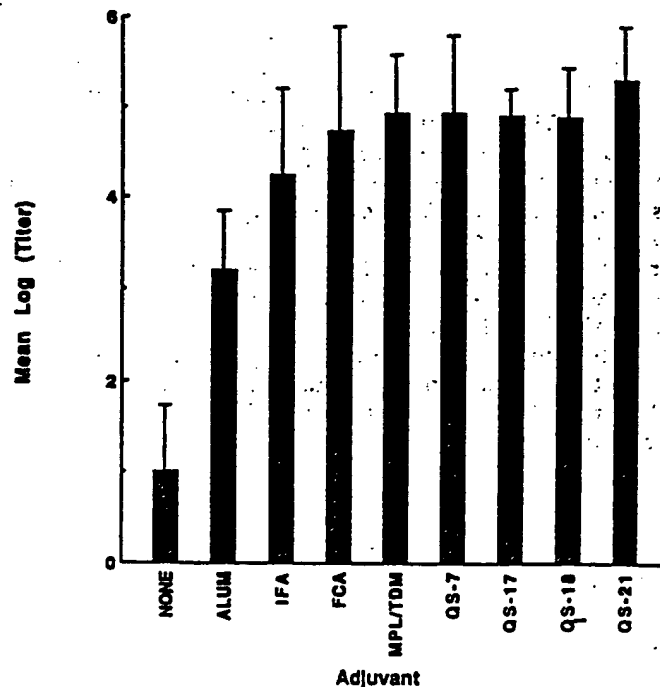


Figure 3. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 10 μ g cytochrome b_5 and the indicated adjuvant. Formulations adjuvanted with CFA and IFA were prepared by emulsification of 100 μ l of Difco CFA or IFA with 100 μ l of a PBS/Ag solution/dose. MPL/TDM formulations were prepared by homogenization of 50 μ g MPL, 50 μ g TDM, 2 μ l Squalene, and 0.2 ml 0.2% Tween 20/PBS/Ag/dose. The alum preparation contained 400 μ g aluminum hydroxide per dose. The saponin preparations, which were fully soluble in aqueous solution, contained 20 μ g of the indicated saponin in 0.2 ml PBS/Ag per dose. Results are expressed as means \pm SD.

TABLE I
Adjuvant effect on Ag-specific IgG Subclass

Adjuvant	Subclass Titer/Total IgG Titer ^a		
	G1	G2 _a	G2 _b
None	1.00	0	0
QS-7	0.35	0.21	0.44
QS-17	0.07	0.21	0.72
QS-18	0.10	0.06	0.84
QS-21	0.15	0.24	0.61
CFA	0.33	0.39	0.27
IFA	0.92	0.07	0.01
Aluminum hydroxide	0.91	0.09	0
MPL + TDM	0.24	0.38	0.38

^a Sera were obtained at day 35 from the cytochrome b_5 immunization study described in Figure 3.

TABLE II
Lethality of saponins to CD-1 mice^a

Dose μ g	Guil-A	QS-7	QS-18	QS-21
125	1/5	0/5	4/5	0/5
250	2/5	0/5	5/5	0/5
500	4/5	0/5	5/5	1/5

^a Results are expressed as number of deaths per group of five mice within 72 h after intradermal injection of saponins.

and 5 μ g of BSA was 4.7 ± 0.13 in comparison with the control group which had a titer of 3.6.

Toxic and hemolytic activities. Toxicity (assessed by lethality) has been associated with the use of saponins as adjuvants (20). In effect, the commercial saponin preparation Guil-A was lethal to mice in the dose range of 100 to 125 μ g (Table II), as determined with one preparation. The purified saponins described here exhibit a wide range of lethalities. QS-18, the predominant saponin species in

the bark from *Q. saponaria* as well as in commercial preparations such as Quil-A. is the most lethal of those tested with deaths observed at doses as low as 25 μ g (data not shown). In contrast, QS-7 is apparently nonlethal up to 500 μ g and QS-21 is lethal only at 500 μ g, with one mouse dead out of five mice receiving this dose (Table II). In mice, the minimum lethal dose/adjuvant-effective dose ratio is 50-fold for QS-21 and even higher for QS-7. However, the QS-18 adjuvant-effective dose is close to the lethal dose when assayed in mice. Apparently, the lethal effects of Quil-A can be explained in part by the large fraction of QS-18, which is the predominant component in its composition. The variability of QS-18 content in the bark used to prepare Quil-A and other commercial preparations will explain the differences in lethality observed with different preparations. From these results, we can state that there is no relationship between relative adjuvant activity and relative lethality.

The hemolytic activities of the purified adjuvant-saponins were compared. Saponins QS-17, 18, and 21 caused hemolysis of SRBC at concentrations as low as 5 to 30 μ g/ml, with concentrations resulting in 50% hemolysis being 25 ± 0 μ g/ml, 15 ± 3 μ g/ml, and 7 ± 2 μ g/ml, respectively (mean and SD of purified preparations derived from two separate bark samples). However, no hemolysis was observed with QS-7 at concentrations up to 200 μ g/ml (highest concentration tested). There is no correlation between hemolytic activity, lethality and adjuvant activity, i.e., QS-7, 18 and 21, have approximately the same adjuvant activity but are widely different in hemolytic activity and lethality.

Carbohydrate composition. Purification of saponins allowed a preliminary structural characterization. The analysis of the composition of the four saponins QS-7, 17, 18, and 21 demonstrated the presence of a highly complex glycoside component, consisting of seven or more monosaccharides in saponin QS-7 and eight or nine monosaccharides in saponin QS-17 (Table III). All four saponins contained components with the same linkage, including terminal rhamnose, xylose, galactose, and glucose residues as well as 3-xylose, 2,3-glucuronic acid, and 3,4-rhamnose (linkage data not shown). It appears that these saponins share a common glycoside structure although there are clear deviations in the carbohydrate composition and linkage of the saponins analyzed.

All saponins contain arabinose except for saponin QS-7. Saponin QS-21 contains a diminished amount of glucose, suggesting that this may be caused by a contaminant as it is present in a ratio significantly lower than 1:1 when normalized to galactose. Monomer size of the predominant saponins was estimated by size exclusion HPLC. For comparison, we used triterpene and triterpene

glycoside standards of known m.w. This analysis was carried out in methanol to prevent micellization. The monomer size ranges from 1800 to 2200 and is consistent with the m.w. predicted for a triterpene with 8 to 10 monosaccharide residues. It is likely that monosaccharides galactose, glucose, and glucuronic acid are each present in a ratio of 1.0 mol of monosaccharide/mol of saponin as higher molar ratios would significantly increase the m.w.

DISCUSSION

These results demonstrate that the saponin fraction obtained by aqueous extraction of *Q. saponaria* bark is actually a heterogeneous group of related glycosides. All previous attempts to purify adjuvant-active *Quillaja* saponins have been in aqueous solution by methods typically used to purify proteins, such as dialysis, ion exchange chromatography, and size exclusion chromatography (7). Although these methods are useful in partially separating saponins from nonsaponin components, they have been ineffective in separating individual saponins because of the tendency of saponins to form mixed micelles. Hence, effective separation requires the use of organic solvents or solvent/water systems that solubilize the amphiphilic saponins as monomers so that the formation of mixed micelles does not interfere with separation. In effect, adsorption and reverse phase chromatography in organic solvents as described in *Materials and Methods* has allowed the purification of individual saponins to a degree of homogeneity that is significantly higher than that achieved by earlier reports (7, 24).

Although previous reports suggested that exposure to organic solvents destroyed adjuvant activity (25), we were able to recover adjuvant activity by using organic solvents for silica and reverse phase chromatography. The carbohydrate analysis of the individual saponins described in this paper indicate that they consist predominantly of one component, although some heterogeneity is still present because multiple linkage forms of individual monosaccharides can be detected. The carbohydrate composition and linkage analysis of the purified *Quillaja* saponins are similar to that determined by Higuchi et al. for the hydrolytic breakdown products isolated from a partially purified *Quillaja* saponin preparation (26). Dalsgaard reported that the saponin fraction isolated by anion exchange and gel filtration (Quil-A) contained the monosaccharides xylose, arabinose, glucose, rhamnose, and fructose (25) in unspecified ratios. None of the saponins described in this study contain fructose. In addition, they contain monosaccharide residues not reported by Dalsgaard (fucose, galactose, and glucuronic acid).

Adjuvant activity was demonstrated to be associated with several of the saponins, including those that appear to be most predominant, QS-7, 17, 18, and 21. Hence, the adjuvant activity of *Quillaja* bark extracts is associated with several distinct saponins rather than a single component, although the carbohydrate analysis indicates that these saponins may be structurally related. Not all peaks contained components that could serve as adjuvants in our test system.

Saponins QS-7, 17, 18, and 21 were tested more extensively because they were the predominant peaks in most bark samples analyzed. These fractions typically induced an increase in Ag-specific IgG titers when used at doses

TABLE III
Molar ratio of monosaccharide/saponin*

Monosaccharide	Saponin			
	QS-7	QS-17	QS-18	QS-21
Rhamnose	2.22	2.34	1.15	1.27
Fucose	0.90	0.96	0.88	0.91
Arabinose	Trace	0.98	0.74	0.77
Xylose	1.28	1.33	1.34	1.44
Galactose	1.00	1.00	1.00	1.00
Glucose	1.35	1.23	1.16	0.35
Glucuronic acid	0.65	0.64	0.72	0.74

* Determined as trimethylsilylated methyl glycosides and normalized to galactose (assumed to be present at 1 mol/mol of saponin).

ranging from 10 to 20 μg in intradermal immunization in mice. The adjuvant effect of these saponins was observed with both BSA and cytochrome b_5 . Evidence that close proximity of Ag and saponin are important for the response was shown by our observation that saponin and BSA injected separately into different flanks of the mice did not induce a boost of Ag-specific IgG titers (data not shown), indicating no apparent systemic response. A similar result has been observed by Bomford (8). The strong antibody response elicited by ISCOM, which are reported to be a complex of saponin, Ag, and lipid (11, 12), are consistent with a close association of Ag and saponin being necessary for the adjuvant response. However, the adjuvant effects of saponins cannot be attributed simply to their detergent properties, i.e., saponin QS-7, which is a poor detergent as revealed by its non-hemolytic properties, has adjuvant characteristics similar to QS-17, 18, or 21, which are highly hemolytic.

Purified saponin adjuvants stimulate an equivalent or higher secondary immune response than that obtained by using aluminum hydroxide, CFA and IFA, or MPL/TDM adjuvants. ELISA titers measured via the end point dilution method, as was done in this study, are thought to be proportional to the concentration of high and medium avidity antibodies (27). Therefore, if it is assumed that the ELISA titers reported here reflect the concentration of these populations, then the purified saponins induce quantities of medium and high avidity IgG comparable with CFA, IFA, and MPL/TDM, and higher than those induced by aluminum hydroxide. However, differences in the concentrations of low avidity antibodies cannot be ruled out. Saponins also influence the Ag-specific isotype profile. A comparison of isotypes produced by mice in response to immunization with purified saponin showed induction of the three major IgG subclasses, G1, G2_b, and G2_a. The isotype profile observed with these purified saponins differs from that reported by Allison and Byars with a crude saponin (2) in which they found predominantly an IgG1 response to immunization of mice with Ag and crude saponin mixture, a response similar to that elicited by aluminum hydroxide. Under the immunization conditions utilized in this study, saponins induced significant levels of IgG2a and IgG2b as well as G1 antibodies; for some saponins, IgG2a predominated. Ag-specific IgE was not detected, even with the highly toxic QS-18, indicating that other components in crude preparations are responsible for the production of reaginic antibodies.

The high level of protection observed with the use of saponins with vaccines in mice (1) may in part be caused by the ability of saponins to induce an isotype profile similar to that observed in natural immunity arising from a viral or bacterial infection. Viral infections in mice induce an IgG response in which IgG2a accounts for 65 to 92% of total specific antibody (28). IgG2a has also been shown to be protective against protozoal infections (29). Both C fixation and antibody-dependent cellular cytotoxicity in mice can be mediated by IgG2a antibodies (30).

Commercially available saponin preparations are highly heterogeneous mixtures of adjuvant-active and inactive components. The relative concentrations of these components will vary according to the source of the bark, leading to difficulty in preparation of batches with a consistent composition. Substantial variation has been

noted between different sources of commercially available saponins (31, 32). Purified saponins can be readily standardized, and this property allows preparation of vaccines with known proportions of a given active saponin or saponins.

The use of purified saponins for immunization allows selection of saponins with the optimal combination of adjuvant activity and negligible lethality. Preliminary studies indicate that some adjuvant-active saponins are significantly more lethal than others when tested at doses over the range of 25 to 500 μg in mice. It may be possible to select an adjuvant-active saponin for use in a vaccine that provides a wider safety margin between adjuvant-active and lethal doses than that in crude saponin extracts (which contains a larger fraction of lethal saponin adjuvants such as QS-18).

No attempt was made to correlate saponin structure with the biologic effects, adjuvant activity, and lethality associated with *Guillaja* saponins. A complete structural determination will involve sequencing of the glycoside moieties, identification of the triterpene, and identification of the point of linkage of the glycoside moieties onto the triterpene backbone. Comparison of the complete structures of naturally occurring variants such as those described here will provide information on what parts of the structure are involved in specific biologic activities. Further information on the minimal structure involved in these activities can be gained by analysis of less complex saponins produced by specific chemical or enzymatic hydrolysis of saponins of known structure. These studies are ongoing.

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CELLULAR AND MOLECULAR IMMUNOLOGY

THIRD EDITION

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and it may therefore play a role in the generation of mucosal immune responses that are mediated by IgA (discussed in Chapter 11).

Interferon- γ

Interferon- γ (IFN- γ), also called immune or type II interferon, is a homodimeric glycoprotein containing two 21 to 24 kD subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains an identical 18 kD polypeptide encoded by the same gene. IFN- γ is produced by activated CD4⁺ and CD8⁺ T cells and by NK cells. Transcription is directly initiated as a consequence of antigen activation and is enhanced by IL-2 and IL-12. IFN- γ produced by natural killer (NK) cells may function as a mediator of innate immunity and contribute to septic shock.

The receptor for IFN- γ is composed of two structurally homologous polypeptides, called IFN- γ R α and IFN- γ R β . Both chains are related to the type I IFN receptor proteins (see Box 12-1). IFN- γ signal transduction is mediated by Jak1, Jak2, and STAT1 α (see Box 12-2). STAT1 knockout mice are completely resistant to IFN- γ actions, as are IFN- γ R α knockout mice.

IFN- γ has several properties related to immunoregulation that separate it functionally from type I IFN:

1. *IFN- γ is a potent activator of mononuclear phagocytes.* IFN- γ directly induces synthesis of the enzymes that mediate the respiratory burst, allowing human macrophages to kill phagocytosed microbes. In murine, but not human, macrophages, IFN- γ acts in concert with TNF or LT to induce the high output isoform of nitric oxide synthase, allowing copious production of NO radicals that have effects similar to those of the reactive oxygen radicals made by human macrophages. In both species, IFN- γ up-regulates the high affinity signaling receptor for IgG, called Fc γ RI (see Chapter 3, Box 3-4). Cytokines that cause such functional changes in mononuclear phagocytes have been called macrophage-activating factors (MAFs). IFN- γ is the principal MAF and provides the means by which T cells activate macrophages. Other MAFs include GM-CSF, and, to a lesser extent, IL-1, TNF, and LT. Macrophage activation is described in more detail in Chapter 13. It is worth noting here that macrophage activation actually involves several different responses, and macrophages are said to be activated when they perform a particular function being assayed. For example, IFN- γ fully activates macrophages to kill phagocytosed microbes but only partially activates macrophages to kill tumor cells.

2. *IFN- γ increases class I MHC molecule expression and, in contrast to type I IFN, also causes a wide variety of cell types to express class II MHC molecules.* Thus, IFN- γ amplifies the recognition phase of the immune response by promoting the

activation of class II-restricted CD4⁺ helper T cells (see Chapter 6, Fig. 6-5). *In vivo*, IFN- γ can enhance both cellular and humoral immune responses through these actions at the recognition phase.

3. *IFN- γ acts on T lymphocytes to promote their differentiation.* As will be discussed later in this Chapter, IFN- γ promotes the differentiation of naive CD4⁺ T cells to the T_H1 subset and inhibits the proliferation of T_H2 cells in mice. These effects may be mediated by activating mononuclear phagocytes to secrete IL-12 and T cells to express functional IL-12 receptors. IFN- γ is also one of the cytokines required for the maturation of CD8⁺ CTLs (see Chapter 13).

4. *IFN- γ acts on B cells to promote switching to the IgG2a and IgG3 subclasses in mice and to inhibit switching to IgG1 and IgE.* The subtypes of IgG induced by IFN- γ are precisely those that bind to Fc γ Rs on phagocytes and NK cells and are also the most potent complement-activating IgG subtypes. Thus IFN- γ induces antibody responses that also participate in phagocyte mediated elimination of microbes.

5. *IFN- γ activates neutrophils, up-regulating their respiratory burst.* It is a less potent activator of neutrophils than TNF or LT.

6. *IFN- γ stimulates the cytolytic activity of NK cells, more so than type I IFN.*

7. *IFN- γ is an activator of vascular endothelial cells, promoting CD4⁺ T lymphocyte adhesion and morphologic alterations that facilitate lymphocyte extravasation.* IFN- γ also potentiates many of the actions of TNF on endothelial cells.

The net effect of these varied activities of IFN- γ is to promote macrophage-rich inflammatory reactions, while inhibiting IgE-dependent eosinophil-rich reactions. Knockout mice in which the IFN- γ or IFN- γ receptor genes have been disrupted show several immunologic defects, including increased susceptibility to infections with intracellular microbes (which cannot be cleared because of defective macrophage activation), reduced production of nitric oxide by macrophages, reduced expression of class II MHC molecules on macrophages after infection with mycobacteria, reduced serum levels of IgG2a and IgG3 antibodies, and defective NK cell function.

Lymphotoxin

Lymphotoxin (LT) is a 21 to 24 kD glycoprotein that is approximately 30 per cent homologous to TNF and competes with TNF for binding to the same cell surface receptors. LT is sometimes called TNF- β . In humans, LT and TNF genes are located in tandem within the MHC on chromosome 6 (see Chapter 5). LT is produced exclusively by activated T lymphocytes and is often produced coordinately with IFN- γ by such cells. Human LT contains one or two N-linked oligosaccharides (accounting for the variability in molecular sizes). The

tsunamis from great earthquakes in the Cascadia subduction zone.

3) Tectonic subsidence during great subduction earthquakes could reconcile rates of short-term uplift with rates of long-term uplift in westernmost Washington. The uplift measured at tide gages and bench marks (2 to 3 mm per year average during the past 50 years) is much faster than that inferred from Pleistocene marine terraces (<0.5 mm per year average during the past ~100,000 years) (18). But these rates need not conflict if, as part of cyclic earthquake-related deformation (19), coseismic subsidence (like that inferred from the buried lowlands) has nearly negated cumulative interseismic uplift (of which tide-gage and bench-mark uplift would be a modern sample).

Jerky Holocene submergence at Washington estuaries thus strengthens the hypothesis that a future great earthquake could emanate from the Cascadia subduction zone. The number and shallow depth of buried lowlands at Willapa Bay (Fig. 3C) may mean that at least six such earthquakes have occurred since sea level approached its present position on mid-latitude coasts, that is, since 7000 years ago (20). The earthquake ruptures, if really from events of magnitude 8 or greater, should have extended coastwise for at least 100 km (21). This corollary can be tested by determining the coastwise extent of individual episodes of coseismic subsidence. Another testable corollary is that shaking during the postulated earthquakes should have caused the liquefaction of Holocene coastal lowland sand (22). If buried lowlands prove coeval for coastwise distances greater than 100 km, and if sand proves to have vented onto some of these lowlands at the start of burial, then the chronology of jerky submergence could be used to constrain the current probability of a great subduction earthquake in the Pacific Northwest.

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Interferon- γ and B Cell Stimulatory Factor-1 Reciprocally Regulate Ig Isotype Production

CLIFFORD M. SNAPPER AND WILLIAM E. PAUL

Gamma interferon (IFN- γ) and B cell stimulatory factor-1 (BSF-1), also known as interleukin-4, are T cell-derived lymphokines that have potent effects on B cell proliferation and differentiation. They are often secreted by distinct T cell clones. It is now shown that IFN- γ stimulates the expression of immunoglobulin (Ig) of the IgG2a isotype and inhibits the production of IgG3, IgG1, IgG2b, and IgE. By contrast, BSF-1 has powerful effects in promoting switching to the expression of IgG1 and IgE but markedly inhibits IgM, IgG3, IgG2a, and IgG2b. These results indicate that BSF-1 and IFN- γ as well as the T cells that produce them may act as reciprocal regulatory agents in the determination of Ig isotype responses. The effects of IFN- γ and BSF-1 on isotype expression are inequivalent.

GAMMA INTERFERON (IFN- γ) PROMOTES the production of immunoglobulin (Ig) by activated murine and human B cells stimulated with interleukin-2 (1) and causes human B cells treated with antibodies to Ig to enter the S phase of the cell cycle (2). Conversely, IFN- γ inhibits the actions of B cell stimulatory factor 1 (BSF-1) on resting B cells, including BSF-1 induction of class II major histocompatibility complex molecule expression (3) and costimulation of proliferation (4). IFN- γ also suppresses the enhancement by BSF-1 of IgG1 and IgE synthesis in B cells stimulated with lipopolysaccharide (LPS) (5). We show here that IFN- γ induces a selective and striking induction of IgG2a production by resting B cells stimulated with LPS in vitro. Furthermore, both IFN- γ and BSF-1 are potent inhibitors of the expression of specific Ig isotypes; IFN- γ blocks IgG3 and IgG2b (6) as well as IgG1 and IgE, whereas BSF-1 blocks IgG3, IgG2b (7), IgG2a, and IgM. These results suggest that IFN- γ and BSF-1 reciprocally regulate Ig isotype production in T cell-dependent immune responses and thus determine many of the biologic consequences of such antibody production. Since BSF-1 and IFN- γ appear to

be produced by separate sets of T cell clones (8), a reciprocal regulatory interaction of T cell subsets may determine Ig isotypic responses to immunization.

Resting B cells were purified from spleens of 8- to 12-week-old DBA/2 mice by incubation with antibodies to Lyr-1, Lyr-2, and Thy 1.2 and complement, followed by Percoll density-gradient centrifugation (4). When these cells were stimulated with LPS, they synthesized large amounts of IgM, considerable IgG3 and IgG2b, and small but detectable amounts of IgG1 and IgG2a (7). Addition of recombinant IFN- γ (rIFN- γ) (10 U/ml) (9) caused a striking increase in IgG2a concentrations and near complete suppression of IgG3, IgG1, and IgG2b production, but had little effect on IgM (Fig. 1). At concentrations of rIFN- γ 30 to 100 times the amount needed to inhibit IgG3, IgG1, and IgG2b completely, suppression of both IgM and IgG2a occurred and could be explained in large part by the striking diminution in viable cell yields at these high rIFN- γ concentrations.

Addition of a hamster monoclonal anti-

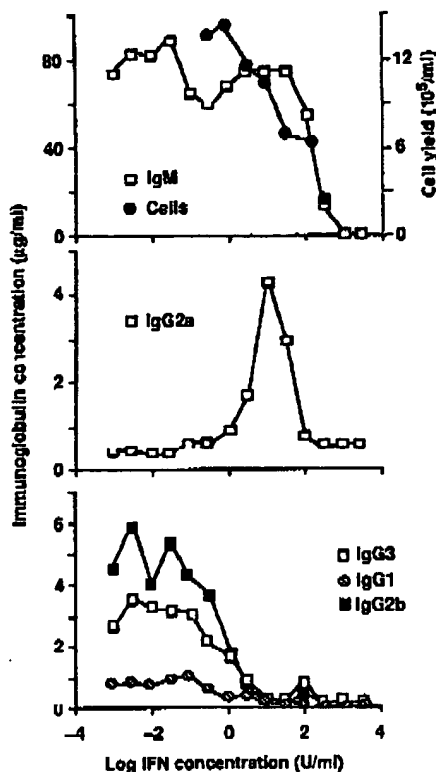
Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

body to rIFN- γ (10) completely reversed both the enhancing and suppressive effects of rIFN- γ on isotypes of Ig secreted by LPS-stimulated B cells, clearly demonstrating that these effects are due to IFN- γ itself and not to some possible contaminant in the rIFN- γ preparation (Fig. 2). The antibody to rIFN- γ had no effect on the concentrations of secreted isotypes of B cells treated with LPS only and by itself did not score in the enzyme-linked immunosorbent assay (ELISA).

The effect of rIFN- γ in enhancing IgG2a production could be achieved by treatment of resting B cells before the addition of LPS. Resting B cells incubated for 48 hours in the presence or absence of rIFN- γ (20 U/ml) were rigorously washed, and equal numbers of viable cells were cultured in the presence of LPS or LPS and antibody to rIFN- γ . Cells incubated in rIFN- γ showed a tenfold increase in the concentration of secreted IgG2a compared to cells incubated in medium alone (Table 1). These cells also showed a three- to fourfold suppression of IgG3. Cells treated with rIFN- γ showed only a modest increase in viable cell yields compared to the group treated with medium, too small an increase to explain the striking increase in IgG2a. Similar results were obtained in the presence or absence of antibody to rIFN- γ in the "secondary" cultures, indicating that rIFN- γ was not significantly carried over into these cultures after washing. We showed earlier that BSF-1 can act on resting B cells to prepare them to secrete IgG1 upon subsequent stimulation with LPS (11). Therefore, both IFN- γ and BSF-1 can act on the resting B cell to specifically regulate Ig isotype secretion upon subsequent addition of LPS.

BSF-1 can cause a 90 to 95% inhibition in the production of IgM, IgG3, IgG2b, and IgG2a in response to LPS, with only modest diminution in cell yield (7, 12). The capacity of rIFN- γ to enhance IgG2a production appears to be independent of this inhibitory effect of BSF-1, since the relative rIFN- γ -induced increase in IgG2a concentrations and the rIFN- γ concentration required for induction are the same over a range of recombinant BSF-1 (rBSF-1) (13) concentrations that progressively inhibit IgG2a secretion (Fig. 3).

The inhibitory effects of rIFN- γ on IgE and IgG1 also appear to be independent of the stimulatory actions of rBSF-1 (Fig. 4). Thus, the concentrations of rIFN- γ required to inhibit IgE and IgG1 are the same for a wide range of rBSF-1 concentrations. In particular, IgE concentrations can be moderately or strikingly enhanced by rBSF-1, at 600 or 10,000 U/ml, respectively, and IgG1 levels show a bimodal stimulatory response



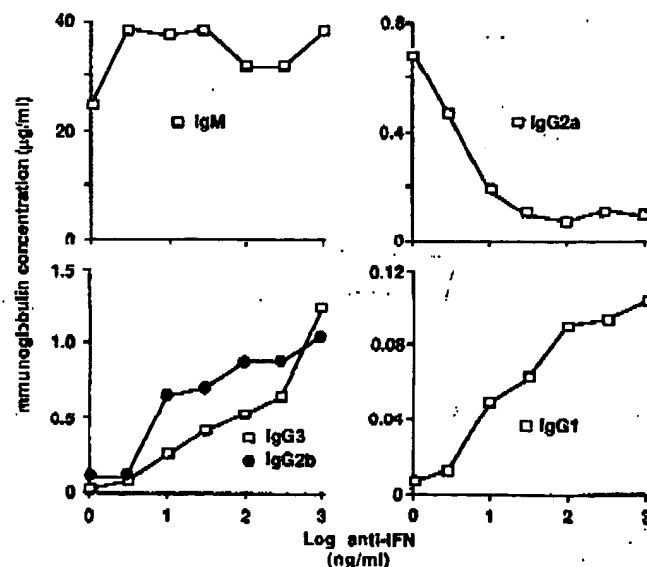
to rBSF-1, with peaks at 125 and 10,000 U/ml. The latter effect is highly reproducible in detailed concentration-response titration and will be reported separately (12). The inhibitory effects of rIFN- γ are essentially the same in each of these groups and cannot be explained by diminution of cell yield, since IgG1 and IgE production are almost completely inhibited by rIFN- γ at 10 U/ml, whereas >100 U/ml is required for comparable inhibition of cell yields (Fig. 1).

Further support for the independence of action of rBSF-1 and rIFN- γ on switching to IgG1 production is shown by the finding

Fig. 1. IFN- γ modulates Ig isotype secretion by LPS-activated B cells. Purified resting B cells were obtained by discontinuous Percoll gradient centrifugation and were distributed into 96-well microtiter plates at 1×10^5 cells in 200 μ l of culture medium (RPMI 1640, 10% fetal bovine serum, γ -glutamine (2 mM), 2-mercaptoethanol (0.05 mM), penicillin (50 μ g/ml), and streptomycin (50 μ g/ml)). Cells were then stimulated with LPS (20 μ g/ml) and increasing concentrations of murine recombinant IFN- γ prepared in Chinese hamster ovary cells. Cultures were carried for 8 days at 37°C in a 6% CO₂ atmosphere, after which culture supernatants were removed for analysis of Ig isotypes by a solid-phase immunoassay (ELISA) (12) and for determination of viable cell yield. The ELISA assay, in which a fluorescent product was generated by specifically bound alkaline phosphatase-conjugated antibodies acting on the substrate methyl umbelliferyl phosphate, specifically detected individual Ig isotypes even when high concentrations of other Ig isotypes were present in the culture supernatants. Cultures were established in duplicate for each point. Results are reported as the mean Ig isotype concentration. For the data points presented in Figs. 1 to 4, the mean relative standard error (\pm SD) is 0.113 ± 0.089 .

that rIFN- γ can fully inhibit IgG1 production when added after rBSF-1 has been removed from culture. In these experiments, B cells were incubated with medium or rBSF-1 for 48 hours, washed, and stimulated with LPS with or without rIFN- γ . Cells treated with rBSF-1 produced 6.5 μ g per milliliter of IgG1 on subsequent stimulation with LPS, a 12-fold enhancement in IgG1 secretion over those treated in medium only. Addition of rIFN- γ and LPS at the same time reduced IgG1 production to 0.3 μ g/ml in the group treated with BSF-1. The concentrations of rIFN- γ used (20 U/ml) did not diminish IgM production, an indication that this "late" inhibitory effect of rIFN- γ on IgG1 production could not be explained by inhibition of cell growth.

Fig. 2. Antibody to IFN- γ (anti-IFN- γ) reverses the effect of IFN- γ on LPS-stimulated murine B cells. Purified resting B cells were stimulated with LPS (20 μ g/ml) and recombinant IFN- γ (20 U/ml) in the presence of increasing concentrations of purified monoclonal anti-IFN- γ (10). Culture supernatants were removed after 6 days for measurement of Ig isotype concentrations by ELISA.



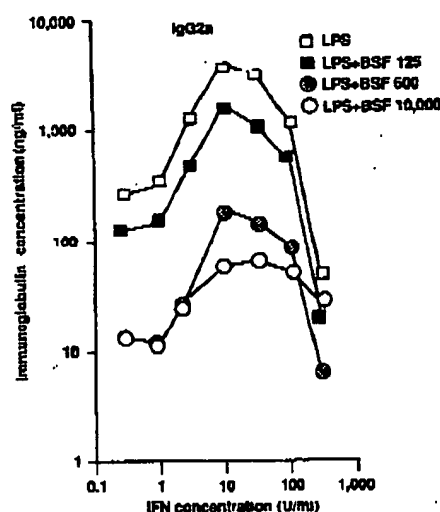


Fig. 3. IFN- γ enhancement of IgG2a secretion is independent of BSF-1 action. Purified resting B cells were stimulated with LPS (20 μ g/ml), recombinant BSF-1 at 0, 125, 600, and 10,000 U/ml, and increasing concentrations of recombinant IFN- γ . Ten units of rBSF-1 is approximately equal in activity to 1 U of T cell-derived BSF-1, measured as described by Ohara *et al.* (13). Culture supernatants were removed after 6 days for measurement of IgG2a concentrations by ELISA.

Our results indicate that both rIFN- γ and rBSF-1 promote the expression of specific Ig isotypes while inhibiting the expression of other isotypes. Recent studies suggest these reciprocal regulatory effects may also operate in vivo. Antibody to BSF-1 blocks IgE production by helminth-infected mice (14) and IFN- γ enhances serum IgG2a concentration in mice treated with antibody to IgD (15). Although the mechanism through which IFN- γ exerts its stimulatory effects on IgG2a production has not been established, it seems likely that it, like BSF-1, acts to cause Ig class switching. BSF-1 added prior to (13) or together with LPS (7) enhances IgG1 production by B cells selected for the absence of membrane IgG expression.

The demonstration that the production of IFN- γ and of BSF-1 are largely segregated in cloned T cell lines (8) suggests that the distinctive Ig isotype regulatory patterns of

these lymphokines will also be true of the cells that secrete them. It has been proposed that IFN- γ -producing T cells be designated T_H1 cells; these cells also secrete interleukin-2. By contrast T cell clones that secrete BSF-1 have been designated T_H2 cells; these cells fail to produce either IFN- γ or interleukin-2.

The effect of IFN- γ on IgG2a production is particularly interesting in view of the capacity of IFN- γ to enhance the expression of human Fc receptors analogous to the mouse FcR1 receptor specific for IgG2a (16). IgG2a antibody is the most effective isotype for the induction of macrophage and killer cell antibody-dependent cellular cytotoxicity (ADCC) of tumor cells, whereas IgG1 has very limited activity in ADCC (17). Furthermore, the marked superiority of IgG2a over IgG1 in complement fixation (18) indicates the striking biologic difference in antibodies of these two isotypes. These findings suggest that IFN- γ may be important in immune responses in which ADCC, opsonization, and complement-mediated lysis play an important protective role. In this regard, it is striking that IgG2a is the predominant IgG isotype in antibody responses to a series of DNA and RNA viruses, in which opsonization and complement-mediated lysis of viruses and destruction of virus-infected cells by ADCC may be of great importance (19). Indeed, the protective effects of antibody in herpes simplex virus infections and its immunotherapeutic role in Friend leukemia virus-induced diseases depend on intact Fc portions (20).

This leads us to propose an evolutionary selection for the production of the IgG isotype most protective against such infections—namely, IgG2a—through the preferential activation of the set of T cells that secrete IFN- γ (T_H1 cells). Although it is beyond the scope of this discussion, we wish to point out that BSF-1 is important in the regulation of IgE production (14), that it induces the expression of IgE-binding Fc receptors on B cells (21) and enhances the growth of mast cells (22). This suggests that

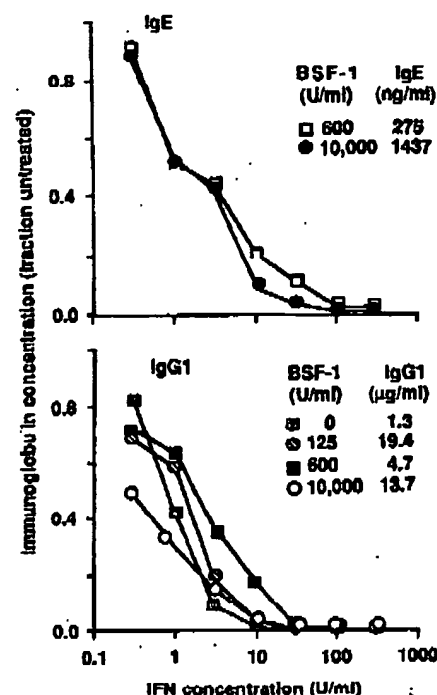


Fig. 4. IFN- γ inhibition of LPS-induced secretion of IgG1 and IgE is independent of BSF-1 action. Purified resting B cells were stimulated with LPS (20 μ g/ml), recombinant BSF-1 (0, 125, 600, and 10,000 U/ml), and increasing concentrations of recombinant IFN- γ . Culture supernatants were removed after 6 days for measurement of IgE and IgG1 concentrations by ELISA. Ig isotype levels are expressed as a fraction of levels obtained in the absence of IFN- γ .

BSF-1 and T_H2 cells may be adapted to responses to certain parasitic agents. The determination of the relative sensitization of T_H1 and T_H2 cells in response to viral infections and to other immunogens will be of critical importance in testing this concept. If T cell subsets have distinctive response patterns, the determination of the properties that cause the activation of cells of these two subsets will be of critical importance in clarifying the regulation of Ig isotype production.

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Table 1. IFN- γ acts on resting B cells to modulate Ig isotype secretion upon subsequent stimulation with LPS. Small resting B cells were preincubated in medium with or without IFN- γ (20 U/ml) for 48 hours. Cells were washed three times and stimulated with LPS (20 μ g/ml) or LPS and anti-IFN- γ (2 μ g/ml) for an additional 6 days. Culture supernatants were removed for measurement of Ig isotype concentrations by ELISA. Each group consists of three replicates. Values reported are mean Ig concentrations; relative standard errors varied between 4% and 17%.

Preincubation	"Secondary" cultures	IgG3 (ng/ml)	IgG2a (ng/ml)	Viable cell yield (10^5 /ml)
Medium	LPS	875	74	11.2
	LPS + anti-IFN- γ	587	27	12.0
IFN- γ	LPS	230	250	14.2
	LPS + anti-IFN- γ	215	287	14.0

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Electrophoretic Evidence for Genetic Diploidy in the Bracken Fern (*Pteridium aquilinum*)

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Analysis of isozyme variability demonstrates that bracken fern (*Pteridium aquilinum*) has a diploid genetic system and expresses solely disomic inheritance patterns. Electrophoretic data indicate that genetically variable progeny are produced in natural populations after intergametophytic mating rather than by a process involving recombination between duplicated unlinked loci. Although some enzymes are encoded by more than one locus, this has resulted from subcellular compartmentalization of isozymes, and there is no evidence of extensive gene duplication resulting from polyploidy. The conclusions reached in this report differ from those which propose polyploidy as an adaptive mechanism for maintaining genetic variability in *Pteridium* and other homosporous pteridophytes.

THERE ARE TWO OPPOSING THEORIES to explain the production of genetically variable offspring in homosporous pteridophytes. The more conventional theory proposes that outcrossing generates heterozygosity and subsequent meiotic segregation leads to variable progeny (1-4). However, the unique life-cycle characteristics of homosporous pteridophytes inspired Klekowski and Baker (5) to formulate an alternative theory concerning the genetic behavior of these plants. The homosporous pteridophytes differ from other vascular land plants in producing wholly independent, potentially bisexual gametophytes. If these gametophytes self-fertilize, they give rise to sporophytes that are homozygous at all genetic loci. The potential for this reproductive process led to the proposal that homosporous pteridophytes are primarily inbreeding (5). A second distinctive feature of homosporous pteridophytes is that they typically have high chromosome numbers, suggesting that they are highly polyploid. According to Klekowski and Baker (5), such polyploidy could be a genetic adaptation required to overcome the extreme homozygosity imposed by recurrent inbreeding. To release variability from these homologically homozygous plants, Klekowski proposed that homoeologous chro-

mosomes (those from different genomes within a polyploid) pair during meiosis (6). Such pairing would result in recombination between different genomes and the subsequent release of genetic variability among progeny.

Several approaches, including cytological studies (7), segregation of morphological markers (8), and segregation of electrophoretically detectable genetic markers (9), have been used to detect the results of homoeologous pairing. However, more recently, several studies have questioned the universality of Klekowski's hypotheses by demonstrating that some homosporous pteridophytes are outcrossing (2-4) and genetically diploid (10, 11). These studies, however, did not include the species that had been used to demonstrate homoeologous pairing. By means of electrophoretic analysis of enzyme variability, Chapman *et al.* (9) found that in bracken fern (*Pteridium aquilinum*) several enzymes were expressed as multiple bands. Chapman *et al.* (9) interpreted these results as evidence that there were duplications in the coding genes resulting from polyploidy. Furthermore, the variability expressed among siblings was attributed to homoeologous recombination in the parental generations.

Although these results seemed conclusive

at the time, it has been shown that multiple-banded isozyme patterns do not always indicate polyploidy. Use of an inappropriate grinding buffer during preparation can lead to enzyme breakdown and subsequent ghost banding or poor band resolution (12). Multiple bands can also result from assaying enzymes composed of more than one subunit. For example, a dimeric enzyme appears as one band in homozygotes but as three bands in heterozygotes (13). Also, many diploid plants have been shown to express several isozymes for certain enzymes, each isozyme encoded by a separate genetic locus within a single genome (14). These isozymes are active in different compartments (for example, chloroplasts or cytosol) within the cell (15). Multimeric isozymes and enzyme compartmentalization were not discussed by Chapman *et al.*, and the grinding buffer they used was a simple one (9), which may have resulted in enzyme breakdown.

The present study reassesses the inheritance patterns of polymorphic structural genes in *P. aquilinum* by means of updated protocols for horizontal starch gel electrophoresis (16). This study also differs from that of Chapman *et al.* (9) in that we directly analyzed gametophytic progeny (representing individual meiotic products), rather than examining sporophytes arising from self-fertilized gametophytes. Thirty-nine single-frond spore samples were collected from wild sporophytes across the United States, Mexico, and Europe (17). Each spore sample was sown separately onto nutrient agar medium (18) and cultured under standard conditions (19). When gametophytes were 3 weeks old, prior to maturation of gametangia, they were harvested for electrophoretic analysis. At least ten gametophytes from each spore sample (giving a total of

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